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Phylogenetics, systematics and evolution of the temperate woody bamboos with an emphasis on the Kuruna clade

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Phylogenetics, systematics and evolution of the temperate woody bamboos with an emphasis on the *Kuruna* clade

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ABSTRACT

The subfamily Bambusoideae (bamboos) is one of 12 subfamilies in Poaceae (grass family) and is primarily associated with forest habitats. Bambusoideae, which include nearly 1,500 species worldwide, is classified into two tribes of woody bamboos (the tropical Bambuseae and the temperate Arundinarieae) and one tribe of herbaceous bamboos (the Olyreae). The Arundinarieae, with ca. 550 species, is well known for its taxonomic and phylogenetic complexity; to date, there are twelve major lineages found in Arundinarieae based on analyses of selected chloroplast DNA (cpDNA) markers, nuclear DNA genes and complete plastomes. The main objectives of this dissertation are to: 1) conduct molecular phylogenetic analysis for the temperate woody bamboo species using chloroplast DNA with particular emphases on testing the monophyly of Sri Lankan temperate woody bamboos, placing them in the correct genus or genera and perform a taxonomic revision for this group; 2) understand the phylogenetic relationships among and within the woody bamboo clades using complete plastome sequences and low-copy nuclear markers and evaluate morphological evolution of some important characters such as rhizomes and pseudospikelets; 3) develop a web-based multi-access interactive key for the Sri Lankan and South Indian temperate woody bamboos; 4) understand the population genetic structure of *A. debilis*, a highly threatened temperate woody bamboo species.

The twelfth lineage of the Arundinarieae, the *Kuruna* clade distributed in Sri Lanka and south India, is based on five plastid markers (*rps16–trnQ, trnC–rpoB, trnD–trnT*, *trnT–trnL* and *ndhF 3'*). In addition to the recognition of this clade as a new genus, I generated a monograph for *Kuruna*, treating the six Sri Lankan *Kuruna* species: *K.*

debilis, *K. densifolia*, *K. floribunda*, *K. scandens* and *K. walkeriana* plus the newly described *K. serrulata*, and the south Indian endemic *K. wightiana*. The seven species are described and illustrated and a dichotomous identification key is provided; a preliminary conservation status assessment for each species is also included. I also explored the phylogenetic relationships among the twelve major lineages of Arundinarieae based on sequences of both complete plastomes and 3 low-copy nuclear genes. Most of the previously recognized major clades were supported by both data sets, though there were some conflicting phylogenetic signals. Furthermore, I designed a web-based multi-access identification tool (WEBiKEY) and tested for its usability with genus *Kuruna* as a sample dataset. This simple, easy-to-use interactive key enables users with plant material from an unknown species in *Kuruna* to visually inspect characteristics of the bamboo and identify it as one of the seven species in the genus.

I also report the results of a preliminary analysis of genetic diversity and population structure in six known Sri Lankan populations of *Kuruna debilis* in Sri Lanka. Even though the sample size was relatively low, allelic diversity was high at most loci and, given the limited distances separating populations (<65 km apart), they exhibited fairly high genetic differentiation (F_{ST} = 0.113) and strong isolation by distance. STRUCTURE, neighbor-joining, and Neighbor-Net analyses agree that the six *K. debilis* populations group into three genetic clusters consistent with the spatial proximity of populations. As the first population genetics study of Bambusoideae in Sri Lanka, I anticipate that our results will provide a foundation for future comparative population genetics and conservation studies in the country.

CHAPTER 1

GENERAL INTRODUCTION

Organization of the Thesis

This dissertation focuses on the phylogenetic relationships among the temperate woody bamboo (Poaceae: Bambusoideae: Arundinarieae) clades with an emphasis on Sri Lankan temperate woody bamboos and their morphology and population genetics. The initial motivation for this study was to better understand the relationships within and among the Sri Lankan temperate woody bamboos, because the most recent study of Sri Lankan bamboo diversity (Soderstrom and Ellis 1988) was published over two decades ago, before the development of molecular techniques. Soderstrom and Ellis (1988) provided detailed morphological and anatomical descriptions and illustrations, but did not examine any aspects of population genetics. Further, recent molecular phylogenetic analysis of the temperate woody bamboos suggested that at least some of the temperate woody bamboos in Sri Lanka, India, Africa and Madagascar are likely to be critical to understanding the early evolution of the diverse temperate bamboo lineage (Triplett & Clark 2010, Stapleton 2013, Ma et al. 2014). In light of these results, it was also evident that the generic classification of the Sri Lankan species needed to be updated. Therefore, to understand the fundamental questions surrounding these species, it is essential to have a robust phylogeny-based understanding of diversity within this lineage.

This dissertation is organized into an introductory chapter (Chapter 1), five main data chapters (Chapters 2–6), and a general conclusions chapter (Chapter 7). This first chapter includes a literature review of the taxonomic history and morphological and

molecular systematics of the temperate woody bamboos. The second chapter comprises a paper entitled “A new genus and a major temperate bamboo lineage of the Arundinarieae (Poaceae: Bambusoideae) from Sri Lanka based on a multi-locus plastid phylogeny” published in *Phytotaxa* (Attigala et al. 2014). The focus of this study was a phylogenetic analysis of the temperate woody bamboo clade with an emphasis on the Sri Lankan woody bamboos based on chloroplast sequence data from five markers. The Sri Lankan temperate woody bamboos were recovered as a major lineage in the Arundinarieae and named as the genus *Kuruna*. Chapter 3, entitled “Taxonomic revision of the temperate woody bamboo genus *Kuruna* (Poaceae: Bambusoideae: Arundinarieae)”, consists of a monograph of this genus. This manuscript will be published in *Systematic Botany* and is currently in press. Chapter 4 is entitled “Phylogenetic reconstruction of Arundinarieae (Bambusoideae: Poaceae) based on plastome phylogenomic and low-copy nuclear gene analyses”, and presents a thorough investigation of the relationships among the major lineages of the temperate woody bamboo clade. This manuscript will be submitted to *Molecular Phylogenetics and Evolution*. Chapter 5, entitled “Simple web-based interactive key development software (WEBiKEY) and an example key for *Kuruna* (Poaceae: Bambusoideae)”, presents a web-based interactive key for *Kuruna* that can be easily used by any interested parties such as researchers, ecologists, gardeners, students, etc. This manuscript was submitted to *Applications in Plant Sciences* and the key will be available on-line through the College of Agriculture and Life Sciences. Chapter 6 is a manuscript entitled “Genetic diversity and population structure of the threatened temperate woody bamboo *Kuruna debilis* (Poaceae: Bambusoideae: Arundinarieae) from Sri Lanka based on microsatellite analysis” which presents an analysis of the population-

level genetic structuring of this species. This manuscript was submitted to the *Journal of the National Science Foundation of Sri Lanka*. The final chapter summarizes the major findings of this dissertation along with a discussion of some future research directions.

Literature Review

The subfamily Bambusoideae (bamboos), which includes nearly 1,500 species, is one of the 12 subfamilies of Poaceae (grass family) and is primarily associated with forest habitats (Clark et al. 2015). This represents the only major lineage of grasses to diversify in the forests, although a few taxa have adapted to other habitats. Bamboos are important components of forest and tropical high altitude grassland ecosystems worldwide (Soderstrom & Calderón 1979, Judziewicz et al. 1999, Bamboo Phylogeny Group [BPG] 2012, Clark et al. 2015). The human consumption of bamboos dates back to early in human history and bamboo is also used in numerous ways in everyday life for shelter, fuel, transportation, food, musical instruments, arts, craft, and medicines (Judziewicz et al. 1999, Shaanker et al. 2004). Many studies, both morphological and molecular, support the monophyly of Bambusoideae and the presence of strongly asymmetrically invaginated arm cells as seen in cross section is the structural synapomorphy that differentiates this subfamily (Zhang & Clark 2000, BPG 2012). Approximately a third of bamboo species occur in north temperate regions (Asia, North America, and high elevation habitats of Africa, Madagascar, India and Sri Lanka), a third occur in the paleotropics, and a third occur in the neotropics (Judziewicz & Clark 2007).

Tribal classification of the Bambusoideae

Recent molecular phylogenetic studies have placed Bambusoideae in the BEP (Bambusoideae, Ehrhartoideae, Pooideae) clade of Poaceae (Grass Phylogeny Working Group [GPWG] 2001, Bouchenak-Khelladi et al. 2010, Zhang 2011, GPWG II 2012, Wu & Ge 2012). Bambusoideae consist of three tribes: tropical woody bamboos (Bambuseae, ca. 812 species), temperate woody bamboos (Arundinarieae, ca. 546 spp.) and herbaceous bamboos (Olyreae, ca. 124 species) (Clark et al. 2015). The monophyly of each of these three tribes is well -supported (Sungkaew et al. 2009, Bouchenak-Khelladi et al. 2010, Triplett & Clark 2010, Kelchner et al. 2013, Wysocki et al. 2015), but the relationships among them remain uncertain. Analyses based on cpDNA markers or complete plastome sequence data are consistent in recovering a well-supported sister relationship between Bambuseae and Olyreae, with the Arundinarieae sister to that clade, although Kelchner et al. (2013) could not rule out monophyly of the woody bamboos (Sungkaew et al. 2009, Kelchner et al. 2013, Wysocki et al. 2015). However, a recent study based on three low-copy nuclear markers supports monophyly of the woody bamboos (Triplett et al. 2014) but also infers a complex history of hybridization and allopolyploidy for each of the woody tribes.

Woody bamboos, both Bambuseae and Arundinarieae, differ from the Olyreae by having culm leaves (leaves modified for the protection and support of the tender young shoots), complex vegetative branching, an outer ligule on the foliage leaves, bisexual flowers and usually gregarious monocarpy (with flowering cycles ranging from a few years to 120 years) (Judziewicz & Clark 1997, Judziewicz et al. 1999, BPG 2012). Conversely, the Olyreae lack an outer ligule and usually lacks differentiated culm leaves,

and have limited vegetative branching, usually nearly continuous or seasonal flowering, and unisexual spikelets. Furthermore, all Olyreae except *Buergersiochloa* (an endemic from New Guinea) have crenate (olyroid) silica bodies (Soderstrom & Ellis 1987, Zhang & Clark 2000, Clark et al. 2007, BPG 2012).

Taxonomic history classification and significance of Arundinarieae

Of the three tribes of Bambusoideae, Arundinarieae is well known for its controversial and difficult taxonomy (Triplett et al. 2014) and many of the classifications based only on morphology or anatomy are incongruent (Li 1997, BPG 2012). Different woody bamboo classification systems treat the temperate woody bamboos differently (Keng 1959, McClure 1966, Keng 1982a b 1983a, Clayton & Renvoize 1986, Soderstrom & Ellis 1987, Dransfield & Widjaja 1995, Keng & Wang 1996, Li 1997, Ohrnberger 1999). By the end of the 1990s, members of the currently recognized Arundinarieae were classified into three subtribes, the Arundinariinae, Shibataeinae and Thamnocalaminae, based on rhizome structure (pachymorph vs. leptomorph) and the presence or absence of pseudospikelets.

Even though clear morphological synapomorphies have yet to be identified, the monophyly of the temperate woody bamboos is strongly supported by many molecular studies (references cited in BPG 2012, Kelchner et al. 2013, Attigala et al. 2014, Ma et al. 2014). The temperate woody bamboos are recognized morphologically by the presence of leptomorph, monopodial rhizomes (pachymorph in some species), basipetal vegetative branch development and tetraploidy (2n=48) (BPG 2012). However, the evident polyphyly of these three subtribes as seen in molecular analyses led researchers to use numbered lineages within Arundinarieae; currently there are 12 major lineages

recognized within the tribe (Triplett & Clark 2010, Zeng et al. 2010, Yang et al. 2013, Attigala et al. 2014) (Figure 1), with the twelfth lineage being contributed by the work presented in this dissertation.

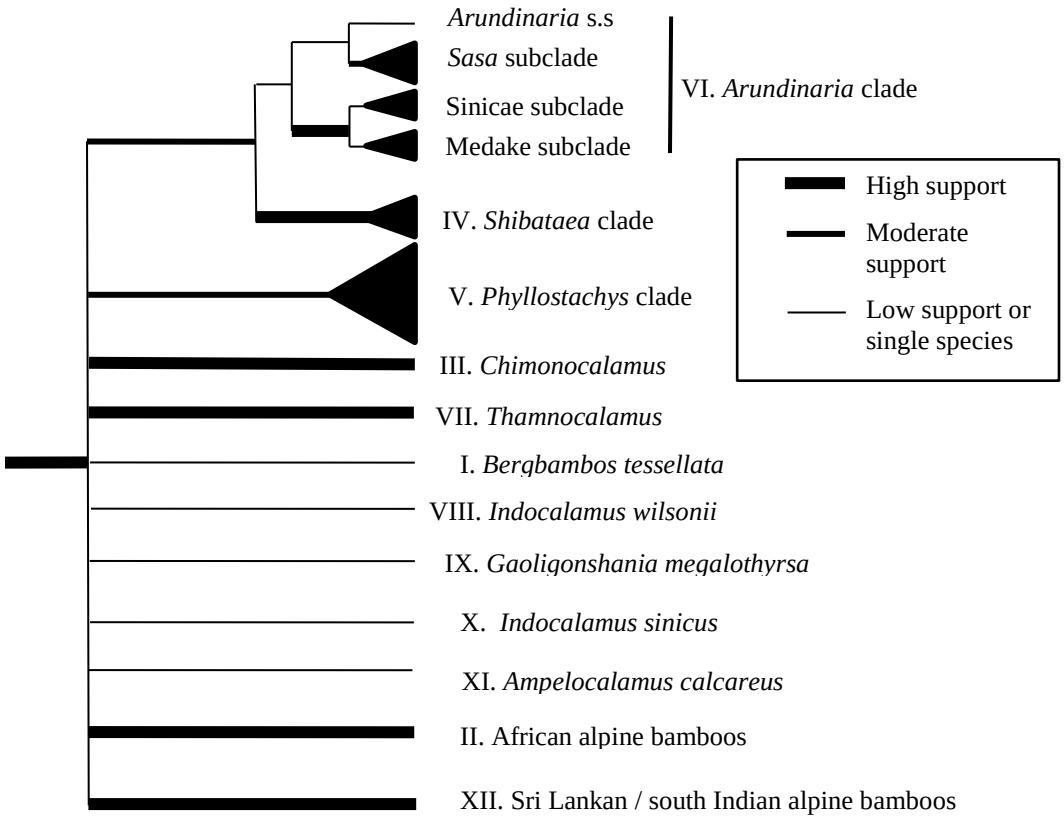

Figure1. Summary phylogenetic tree based on 12 cpDNA + 2 nuclear regions shows the twelve major lineages in Arundinarieae (Zeng et al. 2010, Triplett & Clark 2010, Yang et al. 2013, Attigala et al. 2014).

The difficulty in obtaining resolution among these twelve lineages of Arundinarieae could be due to a many evolutionary phenomena including recent and rapid radiation, convergent evolution, intergeneric hybridization, and lower rate of DNA sequence evolution (Triplett & Clark 2010; Zeng et al. 2010; Zhang et al. 2012; Yang et al. 2013; Ma et al. 2014; Triplett et al. 2014) associated with the long generation times (Janzen 1976). Though the relationships among these lineages were unresolved at the

time this dissertation work started, a recent study revealed some resolution among the Arundinarieae clades (Ma et al. 2014) and on-going independent work by a Chinese group and by me and our collaborators has led to better resolution of lineages within Arundinarieae. Within the tribe, a strongly uneven distribution of species among the clades is evident, with majority of the species clustered into only two lineages: the *Arundinaria* clade (clade VI) and the *Phyllostachys* clade (clade V), the other clades being monotypic or with only a few species (Triplett & Clark 2010, Zeng et al. 2010, Yang et al. 2013, Attigala et al. 2014).

Bamboo, in general, is an economically, culturally and ecologically important plant worldwide. There is an enormous economic significance associated with the members of the Arundinarieae. Like the tropical woody bamboos, the temperate woody bamboos are also used by mankind for thousands of years (Liese et al. 2015) in countless ways, such as for food and beverages, construction materials, ornamentals, musical instruments, etc. For example, different species of temperate woody bamboo *Phyllostachys* are used as a commercial source for bamboo shoots (Rubatzky & Yamaguchi 1997) for hundreds of years. Also, there is substantial amount of ecological importance associated with this tribe. These temperate woody bamboos are important in temperate montane forests and high altitude montane grasslands (Clark et al. 2015 & others cited therein). Majority of the tribe possess leptomorph rhizomes and play a key role in forest dynamics due to their colonizing ability, and gregarious, monocarpic flowering permits forest to reoccupy (Stern 1999). In addition, many animals such as pandas, gorillas, bears, deer, cattle and goats depend on eating these temperate bamboos

(Clark et al. 2015, Pers. Obs.). The tight evolutionary relationship of pandas with Arundinarieae is remarkable and critical for the survival of pandas (Jin et al. 2011).

Taxonomic history of *Arundinaria*

The genus *Arundinaria* was originally described by Michaux (1803) based on a North American species. Among the temperate bamboos, *Arundinaria* is the oldest generic name and over 400 species have at one time or another been classified within it. In the traditional sense, at least since McClure (1973), the genus also includes East Asian, African and Madagascan species. Soderstrom & Ellis (1988) treated *Arundinaria* in the broad sense and included species from Sri Lanka, India and China. In addition, according to Soderstrom & Ellis (1988), the Sri Lankan *Arundinaria* species exhibit similarities with some Chinese and Indian *Arundinaria* species that had been placed by some authors (Yi 1983) in another segregate genus, *Fargesia* Franchet. Other authors have included these species and similar taxa from Africa and Madagascar in the genus *Yushania* P.-C. Keng (Majumdar 1989). Based on recent molecular phylogenetic studies, *Arundinaria* s.s. is now restricted to the three North American species: *A. gigantea* (Walter) Muhl. (type species), *A. tecta* (Walter) Muhl. and *A. appalachiana* Triplett, Weakley & Clark (Triplett & Clark 2010). Thus, the generic classification of the Sri Lankan temperate woody bamboo species must be updated in light of this recent work.

Native bamboos of Sri Lanka and Southern India

Sri Lanka and the Western Ghats of India are considered as a single biodiversity hotspot due to their shared biogeographical history. Myers et al. (2000) show that the moist rain forests of the Western Ghats of peninsular India and the rain forests of south-

west Sri Lanka together form a refugium of the relict biota of the former Indian plate: a plate that gradually isolated from other continents for a period of over 25 million years in the mid-Paleocene to late Eocene era (60–35 million years). However, Sri Lanka remained in full contact with India until the last major sea level rise 6000 years ago, which separated these two countries by the narrow and shallow Palk Strait (McLoughlin 2001).

The indigenous flora of Sri Lanka has about 7,000 species of mosses, ferns and flowering plants (Abeywicrama 1986) and nearly a quarter of the angiosperms of Sri Lanka are endemic and highly concentrated in the humid southwestern quarter of the country, which includes moist low country and the montane zone (Gunatilleke & Gunatilleke 1990). Bamboos occur naturally in all three major climatic zones (wet, dry and intermediate zones) in Sri Lanka but no native bamboo is found in extremely dry areas (Kariyawasam 1998). Bamboo, in general, is an economically and culturally important plant for Sri Lanka (De Zoysa 1994, Gunatilleke et al. 1994). Series of studies have been conducted in Sri Lanka mainly focusing on bamboo reproductive ecology (Ramanayake & Yakandawala 1995, Ramanayake & Yakandawala 1998, Ramanayake & Weerawardene 2003), vegetative propagation (Ramanayake et al. 2001a, Ramanayake et al. 2006) and growth and development (Rajapakse 1992, Ramanayake et al. 2001b). But these studies did not focus on population genetics nor were molecular systematics techniques employed to establish evolutionary relationships and to distinguish generic and species boundaries.

To date, nine named species of woody bamboos have been documented in Sri Lanka (Soderstrom et al. 1987, Soderstrom & Ellis 1988, Attigala et al. 2014, Attigala et

al. in press). Although this is not a large number compared to total bamboo diversity, six of these nine woody bamboos are reported to be endemic to Sri Lanka (Attigala et al. in press). About half of the indigenous bamboos are of the shrubby montane type and not well suited for utility purposes (Kariyawasam 1998) but they are important components of high elevation montane forests or grasslands in Sri Lanka. Of the nine bamboos in Sri Lanka, five species until last year were classified within the temperate woody bamboo genus *Arundinaria*: *A. debilis, A. densifolia, A. floribunda, A. scandens* and *A. walkeriana*; these are now reclassified as species of *Kuruna* (Attigala et al. 2014). All these are wind pollinated perennials as far as is known, each with a very limited distributional range (Soderstrom & Ellis 1988). The remaining native Sri Lankan bamboo species belong to the tropical woody bamboos (Bambuseae), but their classification and relationships remain to be tested.

The most recent study of the native Sri Lankan bamboos (Soderstrom & Ellis 1988) was published over two decades ago, before the development of advanced molecular techniques. These studies provided detailed morphological and anatomical descriptions and illustrations, but did not examine any aspects of phylogenetics or population biology. Hence the use of molecular sequence data allowed us to test hypotheses of relationships, place the Sri Lankan taxa in the appropriate evolutionary context, and update their classification. The population genetics component of the study enabled us to assess species level questions, particularly in native Sri Lankan woody bamboos, and determine how much genetic variation exists in these species which will be useful in planning conservation efforts.

Bamboos play a significant role in the biodiversity of the southern region of the Western Ghats. Based on Wight's collections from Nilgiri, Nees (1834) described the temperate woody bamboo *Arundinaria wightiana*. Later, Munro (1868) described another two species of bamboos, *A. densifolia* and *A. walkeriana*, from southern India following the Michaux (1803) concept of *Arundinaria*. Rao (1914) reported *A. floribunda* from Southern India, which was already described based on Sri Lankan specimens by Thwaites (1864). In a taxonomic revision of species described under *Arundinaria* in Southeast Asia and Africa, Chao & Renvoize (1989) transferred all the *Arundinaria* species of south India and Sri Lanka to the genus *Sinarundinaria*. The generic concept of Chao & Renvoize (1989) was also accepted by Seethalakshmi & Kumar (1998). The generic name "*Sinarundinaria*" was used by Nakai (1925) for two temperate woody bamboo species, *Sinarundinaria nitida* and *S. murielae*, and their descriptions were based only on vegetative morphology. However, with the flowering of *S. murielae* and *S. nitida* in the 1970's and 1993 respectively, Stapleton (1994) recognized *Sinarundinaria* as a synonym of *Fargesia*. Hence the generic name "*Sinarundinaria*" became invalid. Keng's (1959) generic concept of *Yushania* was accepted by Majumdar (1989) in his work and thus he treated all the Indian *Arundinaria* species as *Yushania*. Tewari (1992) followed the generic concepts of Nakai (1925) and recognized *A. floribunda*, *A. wightiana* and *A. walkeriana* of India as species of *Indocalamus*. Further, recent studies indicated that some of the native species so far reported only from Sri Lanka are now found in southern India (Muktesh Kumar 2011). However, none of these southern Indian temperate woody bamboo species were included in any molecular studies and a thorough investigation of Indian temperate woody bamboos might reveal additional endemic taxa.

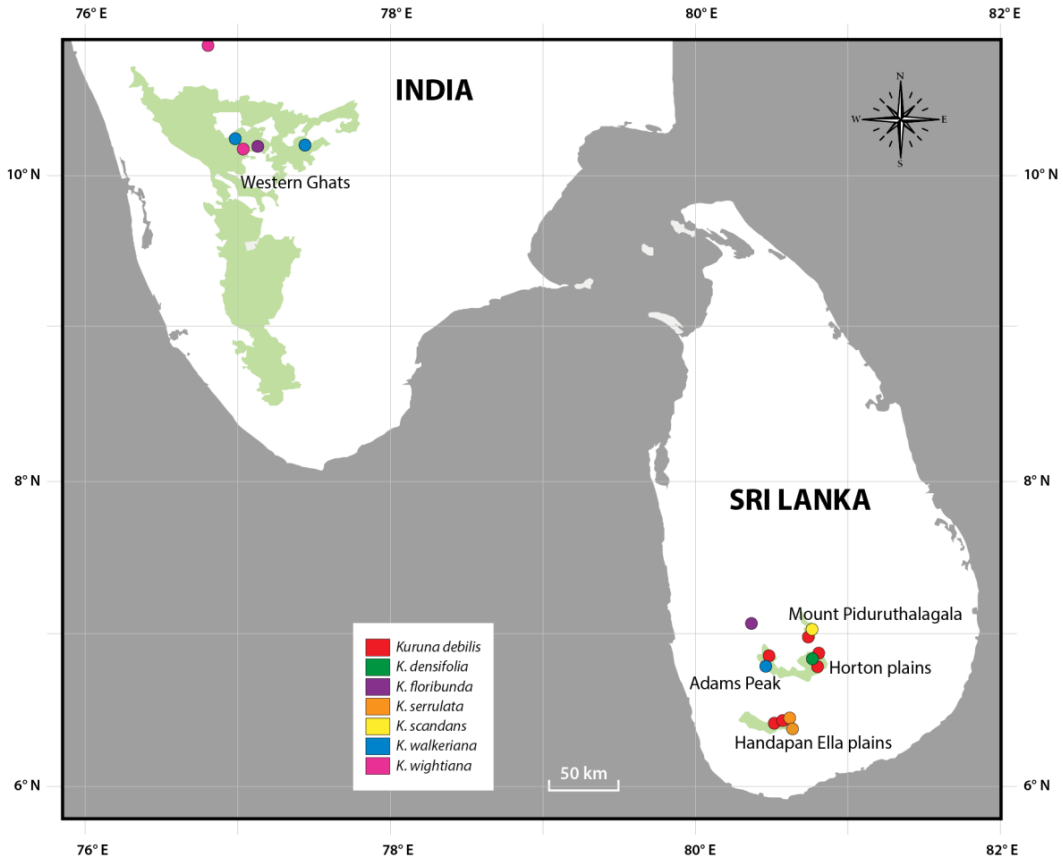

Figure 2. Distribution map of temperate woody bamboos of south India and Sri Lanka.

All these facts summarize our present understanding of the South Asian temperate woody bamboo species, especially from Sri Lanka and India. These groups have never been carefully investigated to understand the relationships of these species with the rest of the temperate woody bamboo clades. Thus a detailed study of these species at both morphological and molecular levels could explain some of the fundamental questions for which we try to seek answers.

Research Objectives

The objectives of this dissertation are to: 1) conduct molecular phylogenetic analyses of the native Sri Lankan *Arundinaria* temperate woody bamboo species using chloroplast DNA with particular emphases on testing their monophyly and placing them in the correct genus or genera; 2) taxonomically revise the temperate woody bamboo species from Sri Lanka and south India; 3) understand the phylogenetic relationships among and within the temperate woody bamboo clades using complete plastome sequences and low-copy nuclear markers and evaluate the morphological evolution of some important structures such as rhizomes and pseudospikelets; 4) develop a web-based multi-access interactive key for the Sri Lankan and south Indian temperate woody bamboos; and 5) understand the population genetic structure of *A. debilis*, a highly threatened temperate woody bamboo species.

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CHAPTER 2

A NEW GENUS AND A MAJOR TEMPERATE BAMBOO LINEAGE OF THE ARUNDINARIEAE (POACEAE: BAMBUSOIDEAE) FROM SRI LANKA BASED ON A MULTI-LOCUS PLASTID PHYLOGENY

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Abstract

Kuruna, a new temperate woody bamboo (Poaceae, Bambusoideae, Arundinarieae) genus from Sri Lanka, is recognized based on chloroplast sequence data from five markers. This genus represents the twelfth major lineage of temperate woody bamboos. Maximum Parsimony, Bayesian Inference and Maximum Likelihood analyses of a combined data set consistently strongly supported the monophyly of this Sri Lankan

temperate woody bamboo clade. Although the Kishino-Hasegawa test is unable to reject the alternative hypothesis of monophyly of the Sri Lankan clade plus *Bergbambos tessellata* from South Africa, *Kuruna* and *Bergbambos* are distinguishable by a combination of morphological characters. A few additional cpDNA markers not previously used in phylogenetic analyses of Arundinarieae were tested to evaluate their utility in this taxonomically difficult tribe.

Introduction

Bamboos are important components of forest and tropical high altitude grassland ecosystems worldwide (Soderstrom & Calderón 1979, Judziewicz *et al.* 1999). The bamboos (Poaceae subfamily Bambusoideae) include approximately 1,450 species (Bamboo Phylogeny Group [BPG] 2012) classified into two tribes of woody bamboos (the tropical Bambuseae and the temperate Arundinarieae) and one tribe of herbaceous bamboos (the Olyreae). Significant animal biodiversity is associated with bamboo-dominated ecosystems (Judziewicz *et al*. 1999, Bystriakova *et al*. 2003, Mutschler & Tan 2003 & others cited in BPG 2012) and bamboos play important roles in forest dynamics (e.g., Li & Xue 1997, Judziewicz *et al*. 1999). Despite the ecological and economic importance of bamboos, basic knowledge of the biology and genetics of woody bamboos is still lacking due in part to their unusual life cycle, with the vegetative phase ranging from a few to 120 years (McClure 1966). Furthermore, the generic classification of bamboos is in a fluctuating state, although the supra-generic classification of bamboo has been improved based on recent phylogenetic analyses (BPG 2012).

Bamboos occur naturally in all three major climatic zones (wet, dry and intermediate) in Sri Lanka and no native bamboo is found in extremely dry areas

(Kariyawasam 1998). Bamboo, in general, is an economically and culturally important plant for Sri Lanka (De Zoysa & Vivekanandan 1994, Gunatilleke *et al*. 1994) and a series of studies have been conducted mainly focusing on bamboo reproductive ecology (Ramanayake & Yakandawala 1995, 1998, Ramanayake & Weerawardene 2003), vegetative propagation (Ramanayake *et al*. 2006) and bamboo growth and development (Ramanayake *et al*. 2001). These studies were carried out before the widespread use of molecular sequence data to establish evolutionary relationships and confirm the generic classification of these species.

Nine species native to Sri Lanka have been documented in Bambuseae and Arundinarieae (Soderstrom & Ellis 1988), eight of which are reported to be endemic (Dassanayake & Fosberg 1994). Of the eight endemic bamboos, five species are classified within the temperate woody bamboo genus *Arundinaria* Michaux (1803: 73) and they are found in high elevation montane forests or grasslands in Sri Lanka. But these shrubby montane-type *Arundinaria* species are not well suited for utility purposes (Kariyawasam 1998). All the Sri Lankan *Arundinaria* species are wind pollinated perennials as far as is known, each with a very limited distributional range (Soderstrom & Ellis 1988) and some are important components of high elevation grasslands in Sri Lanka.

Arundinarieae include ca. 550 species worldwide and are characterized by presence of leptomorph, monopodial rhizomes (pachymorph in some species), basipetal vegetative branch development and tetraploidy (2n=48) (BPG 2012); molecular evidence strongly supports the monophyly of the temperate woody bamboos (BPG 2012, Kelchner *et al*. 2013). Among the temperate bamboos, *Arundinaria* is the oldest generic name and

over 400 species have at one time or another been classified within it. In the traditional sense, at least since McClure (1973), the genus also includes East Asian, African and Madagascan species. Soderstrom & Ellis (1988) treated *Arundinaria* in the broad sense and included the species from Sri Lanka, India and China. In addition, according to the Soderstrom & Ellis (1988) study, all the Sri Lankan *Arundinaria* species exhibited similarities with the Chinese and Indian *Arundinaria* species that had been placed by some authors (Yi 1983) in another segregate genus, *Fargesia* Franchet (1893: 1067). Other authors have included these species and similar taxa from Africa and Madagascar in the genus *Yushania* P.-C. Keng (1957: 355) (Majumdar 1989).

To date, 11 major lineages are found in the temperate woody bamboo clade, mainly based on cpDNA sequence analyses, but the relationships among these clades are still not resolved (Triplett & Clark 2010, Zeng *et al*. 2010, Yang *et al*. 2013). Based on recent phylogenetic studies, *Arundinaria* is now restricted to the three North American species: *A. gigantea* (Walter 1788: 81) Muhlenberg (1813: 14) (type species), *A. tecta* (Walter 1788: 81) Muhlenberg (1813: 14) and *A. appalachiana* Triplett, Weakley & Clark (2006: 88) (Triplett & Clark 2010). Thus, the generic classification of the Sri Lankan species must be updated in light of this recent work.

The primary objective of the current study was to conduct a molecular phylogenetic analysis of the native Sri Lankan *Arundinaria* species using chloroplast DNA sequencing, with a particular emphasis on testing the monophyly of this group and placing them in the correct genus or genera. A detailed morphological comparison of the native Sri Lankan *Arundinaria* species and putatively taxonomically related taxa within Arundinarieae was also conducted to identify distinguishing features for this group. In

addition, several cpDNA markers not previously used in phylogenetic analyses of Arundinarieae were tested to evaluate their utility in resolving relationships among the major temperate bamboo clades.

Materials and Methods

Taxon sampling and outgroup selection

All five known species and a potentially new Sri Lankan *Arundinaria* species were sampled for this study. In addition, representatives from ten of the 11 currently recognized temperate clades were selected based on previous studies (Triplett & Clark 2010, Zeng *et al*. 2010) and their sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Appendix). Sequence data for Clade XI, which includes only *Ampelocalamus calcareus* C.-D. Chu & C.-S. Chao (Chao & Chu 1983: 204) (Yang *et al*. 2013), was not available when this study was conducted and therefore was not included. Several taxa were selected as outgroups based on prior studies (Triplett & Clark 2010, Zeng *et al*. 2010): *Brachyelytrum erectum* (Schreber 1789: 97) Palisot de Beauvois (1812: 155) (Pooideae), *Chusquea spectabilis* L.G. Clark (Fisher *et al*. 2009: 681) (neotropical woody bamboos), *Guadua angustifolia* Kunth (1822: 253) (neotropical woody bamboos) and *Bambusa vulgaris* Schrader ex Wendland (Wendland 1808: 26) (paleotropical woody bamboos).

Chloroplast DNA Marker Selection

Based on prior (Triplett & Clark 2010) and preliminary studies, four intergenic regions (*rps16–trnQ, trnC–rpoB, trnD–trnT, trnT–trnL*) and one coding region (*ndhF* 3' end) that provided reasonable numbers of parsimony-informative characters for

temperate species were selected. An additional set of cpDNA markers, three from domain IV [D4] of chloroplast group II introns (*petD, atpF, ndhA*) and three other intergenic regions (*psbD-trnT, psbJ-petA* and *ycf6-psbM*), based on previous studies (Shaw *et al*. 2005, Shaw *et al*. 2007, Watts *et al*. 2008) were selected to evaluate their potential utility in the temperate clade and in resolving the relationships of the Sri Lankan *Arundinaria* species.

DNA Extraction, Sequencing, Alignment, and Character Coding

Total genomic DNA extractions were performed from silica gel-dried specimens using the Iowa State University DNA Facility's Autogenprep 740 DNA extraction robot. Primers for polymerase chain reactions (PCR) and sequencing protocols of all the markers are listed in Table 1. All PCR and cycle-sequencing reactions were performed in an MJ Research PTC-200 thermal cycler. PCR was performed in 25µL volumes. Amplification products were cleaned using polyethylene glycol (PEG) precipitation to remove unincorporated primers and dNTPs from the PCR products. Sequencing was performed on an ABI 3730xl DNA Analyzer (Perkin-Elmer, Applied Biosystems Division, Norwalk, Connecticut) by the DNA Sequencing Facility at Iowa State University. Automated sequencing output was checked visually for correct automated base-calling. DNA sequences were aligned manually in Se-Al (Rambaut 2001). Gaps introduced with the sequence alignment were later treated as binary, presence/absence characters (Giribert & Wheeler 1999). Autapomorphic, parsimony uninformative indels were not scored, and they were excluded along with other gaps prior to analysis.

TABLE 1. Chloroplast DNA primers used for amplification and sequencing. * indicates published primer sequences that were modified from Triplett & Clark (2010); underlined text indicates modified nucleotide sites. SEQ indicates primers used for sequencing reactions, if different from the PCR primers. § indicates regions that showed very low genetic variation for the temperate clade.

Region	**Primer sequences (5'-3')**	**PCR Parameters**	**Reference**
***ndhF* (3' end)**	972F: GTCTCAATTGGGTTATATGATG 2110R: CCCCCTAYATATTTGATACCTTCTCC SEQ: 1318F*: GGATTAACTGCGTTTTATATGTTTCG 1603R: GCATAGTATTTCCCGTTTCATGAGG	94°C, 1 min; 30x (94°C, 1 min 30 sec; touchdown 53–43°C, 2 min; 72°C, 3 min); 72°C 10 min.	Olmstead & Sweere (1994)
***rps16–trnQ*(1)** (for temperate bamboos)	5' end: 1F: GCACGTTGCTTTCTACCACA 929R: TTCTGTCTACTCGGCTTTCG 3' end: 538F: CGACTCGAATACCAAAAGAGG 1574R: ATCCTTCCGTCCCAGATTTT SEQ: (5') 16Q 650R:GTTCGTTGGATAGAATGGATTC (3') 16Q in-for: GCCGAGTAGACAGAATATATG (3') 16Q 1100R:GGCCAGATTAAAGAATAGGAAG	95°C, 2 min; 35x (95°C, 1 min; 48°C, 10 sec; +17°C, 0.3°C/ sec; 65°C, 5 min); 65°C, 5 min. Note: For some taxa, 628R was used to sequence the 5' amplicon [see rps16–trnQ (2) for primer sequence]	Triplett & Clark (2010)
***rps16–trnQ*(2)** (for all other taxa)	1F: GCACGTTGCTTTCTACCACA 1574R: ATCCTTCCGTCCCAGATTTT SEQ: 334F: CGAGATGGTCAATCCTGAAATG 628R: CTTTTGGTATTCKAGTCGAAG	95°C, 2 min; 35x (95°C, 1 min; 50°C, 10 sec; +15°C, 0.3°C/ sec; 65°C, 5 min); 65°C, 5 min.	Triplett & Clark (2010)
trnC–rpoB	trnC: TGGGGATAAAGGATTTGCAG rpoB*: ATTGTGGACATTCCCTCRTT SEQ: jt400-for: CAGGTCCGAACAGCATTA jt700-rev: CGTAGTAGTAGAATTGCTAG	94°C, 2 min; 35x (96°C, 1 min; touchdown 56–46°C, 2 min; 72°C, 3 min); 72°C, 5m.	PCR: Yamane & Kawahara (2005); SEQ: Triplett & Clark, 2010
trnD–trnT	trnD-for: ACCAATTGAACTACAATCCC trnT-rev: CCCTTTTAACTCAGTGGTA SEQ: trnY-rev: CTCTTTGCTTTGGATCTAG trnE-for: GCCTCCTTGAAAGAGAGATG	94°C, 2 min; 35x (94°C, 45 sec; touchdown 58–48.5°C, 1 min; 72°C, 1 min15 sec); 72°C, 5 min	trnD-for: Demesure *et al.* (1995); trnT-rev, trnY-rev: Triplett & Clark (2010); trnE-for: Doyle *et al.* (1992)
trnT-trnL	trnT-L F: CATTACAAATGCGATGCTCT trnT-L R: TCTACCGATTTCGCCATATC	95°C, 2 min; 35x (95°C, 1 min; 48°C, 10 sec; +17°C, 0.3°C/ sec; 65°C, 5 min); 65°C, 5 min	Taberlet *et al.* (1991)

TABLE 1. continued

Region	Primer sequences (5'-3')	PCR Parameters	Reference
§***atpF intron D4***	sak21F: AAAGGGAGTGTGTGYGAGTT sak22R: CCCGAACCAAAYATGAATCTTTC	80°C, 5min; 35x (65°C, 1min; 0.3°C/s, 50 °C, 1min; 65°C 1.5 min); 65°C, 4 min	Watts *et al.* (2008)
§***ndhA intron D4***	sak26F: CAATATCTCTACGTGYGATTCG sak28R: AACTGTTRGATAATCATAGTCG	80°C, 5m; 35x (65°C, 1min; 0.3°C/s, 50 °C, 1min; 65°C 1.5 min); 65°C, 4 min.	Watts *et al.* (2008)
§***petD intron D4***	sak17F: GGATTATGGGAGTGTRYGACTTG sak18R: CTTTGTTATTGGGATAGGTGAA SEQ: sak19F: GAGACRAYCCANAAAGCA sak18R: CTTTGTTATTGGGATAGGTGAA	80°C, 5min; 35x (65°C, 1min; 0.3°C/s, 50 °C, 1min; 65°C, 1.5 min); 65 °C, 4 min.	Watts *et al.* (2008)
§***psbD-trnT***	psbD: CTC CGT ARC CAG TCA TCC ATA trnT(GGU)-R: CCC TTT TAA CTC AGT GGT AG	80°C, 5min; 30x (95°C, 1min; 50^0C, 0.3°C/s, 50 °C, 1min; 65°C, 4 min); 65°C, 5 min	Shaw *et al.* (2007)
§***psbJ-petA***	psbJ: ATA GGT ACT GTA RCY GGT ATT petA: AAC ART TYG ARA AGG TTC AAT T	80°C, 5min; 30x (95°C, 1min; 50^0C, 1 min, ramp of 0.3°C/s to 65°C; 65°C, 4 min); 65°C, 5 min	Shaw *et al.* (2007)
§***ycf6-psbM***	ycf6F: ATG GAT ATA GTA AGT CTY GCT TGG GC psbMR; ATG GAA GTA AAT ATT CTY GCA TTT ATT GCT	80°C, 5min; 35x (94°C, 1min; 50-55^0C, 1 min, 72°C, 3.5 min); 72°C, 5 min	Shaw *et al.* (2005)

Phylogenetic Analyses

All data were analyzed with Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. MP analyses were conducted using PAUP* 4.0b10 (Swofford 2002), ML analyses were conducted using RaXML version 7.2.6

(Stamatakis 2006), and BI analyses were conducted using MrBayes 3.1 (Ronquist *et al*. 2005).

In MP analyses all characters were treated as unweighted and unordered. Heuristic tree searches were conducted with Tree Bisection and Reconnection (TBR) branch swapping. The initial trees for branch swapping were obtained by 1000 random stepwise taxa addition replicates. To assess the relative support for each node, bootstrap analysis was conducted from 1000 replicates with the heuristic search method and strict consensus trees were calculated. The Akaike information criterion (AIC) calculations, implemented in JmodelTest 0.1 (Guindon & Gascuel 2003, Posada 2008), were used to select the appropriate model of sequence evolution for each DNA partition and for the combined data set excluding indels. ML analyses were conducted using RAXML, version 7.2.6 (Stamatakis 2006), invoking a rapid bootstrap (1000 replicates) analysis and search for the best-scoring Maximum Likelihood Tree with the general time-reversible model of DNA sequence evolution with gamma-distributed rate heterogeneity (the GTRGAMMA model); this was performed for each DNA partition and for the combined data set. BI was conducted with flat priors. The Markov chain Monte-Carlo algorithm was executed for four chains for 10 million generations per run, sampling every 1,000 generations, and a chain-heating temperature of 0.2; this entire procedure was conducted twice. Posterior Probabilities (PP) were analyzed after a burn-in of 10,000 trees and then the remaining samples were summarized and a majority-rule consensus trees were constructed. The BI analyses were conducted for each DNA partition and for the combined dataset. When assessing conflicts among the resultant phylogenies, the threshold value for the bootstrap criterion for both MP and ML was 70% and posterior probability measure for BI was

0.95 (Mason-Gamer & Kellogg 1996, Wilcox *et al*. 2002). Values less than 70% MP Bootstrap/ML Bootstrap and less than 0.95 PP were considered as lacking support.

We tested whether the combined dataset provided sufficient evidence to reject particular hypotheses of relationships suggested by previous morphological studies (e.g., monophyly of the Sri Lankan *Arundinaria* and *Arundinaria* s.s. from North America, monophyly of Sri Lankan *Arundinaria* and other Indian and African temperate bamboo species). Constraint trees were generated in MacClade 4.08 (Maddison & Maddison 2005) by forcing test groups to be monophyletic, but otherwise allowing taxa to "float," and MP analyses were performed in PAUP* using each constraint in turn. The Kishino-Hasegawa (K-H) test (Kishino & Hasegawa 1989) as implemented in PAUP* was then used to test the significance of differences in tree statistics amongst different topologies in comparison with the MP topologies.

In addition, the number of nucleotide substitutions, indels, and inversions (hereafter referred to collectively as Potentially Informative Characters or PICs) (Shaw *et al*. 2007) between the ingroup species and between either ingroup species and the outgroup species were tallied for each cpDNA region to evaluate the potential use of these cpDNA regions in the temperate clade (Shaw *et al*. 2007). The average number of PICs found within each cpDNA region was then computed.

Morphological Comparison

A total of 21 vegetative and reproductive characters were examined in the morphological comparison across *Arundinaria* s.s., Sri Lankan *Arundinaria*, the *Thamnocalamus* Munro (1868: 157) clade, *Bergbambos* and *Oldeania* of the African alpine bamboo clade to understand the differences and similarities among these taxa.

Even though the phylogenetic analyses include both *Oldeania alpina* and *Yushania ambositrensis* (Camus 1913:78) Ohrnberger (1999: 14), the two known taxa of the African alpine bamboo clade, the morphological comparison includes only *Oldeania alpina* due to the lack of good material of *Yushania ambositrensis*. The Sri Lankan *Arundinaria* species were also compared morphologically with *Yushania*, *Chimonobambusa* Makino (1914: 153) and *Indocalamus* Nakai (1925: 148), since some of the Sri Lankan species were included in these genera by previous authors (Nakai 1925, Majumdar 1989). In addition, *Fargesia* was included in this comparison, because Soderstrom & Ellis (1988) discussed morphological resemblances of these Sri Lankan species with the Chinese and Indian *Arundinaria* species that have been placed by some authors (Yi 1983) in *Fargesia*. As *Chimonobambusa* was easily distinguished from the other genera by the combination of leptomorph rhizomes, culms grooved above the basal branches, basal nodes with subequal multiple buds, more or less equal primary branches and pseudospikelets, this genus was not included in the comparison.

The choice of morphological characters for this comparison was based on Stapleton (2013), the characters in the Bamboo Biodiversity website (Bamboo Phylogeny Group 2005), some of which have been used for Bamboo Phylogeny Group morphological phylogenetic analyses, and direct examination of herbarium specimens (held at ISC, K, MO, PDA, US). For each genus, the entire genus was considered whenever possible. For *Fargesia* and *Indocalamus*, the type species of each genus has been included in molecular analyses along with other taxa from each but neither genus as currently delimited is demonstrably monophyletic (e.g., Yang et al. 2013). In addition, many species of *Fargesia* are unknown in flower (Li et al. 2006) and relatively little

herbarium material is accessible for the majority of species in these two genera. We therefore used the type species for each genus as the most appropriate representatives.

Results

Figure 1 shows the average PIC (Potentially Informative Character) values found within each of the 11 tested cpDNA regions. Out of the 11 regions, only *rps16–trnQ*, *trnC–rpoB*, *trnD–trnT*, *trnT–trnL* and *ndhF* 3' gave enough variation in the temperate bamboo clade to provide resolution. The highest percentage average PIC value was 7.82% for *rps16-trnQ* while the second highest was 5% for *trnT-trnL*, whereas the regions *ndhA, ycf6-psbM, atpF, psbJ-petA* had percentage average PIC values less than 1.00% and *petD* and *psbD-trnT* showed no variation. In addition, *trnD-trnT, ndhF* (3' end) and *trnC-rpoB* had very similar percentage average PIC values: 3.37%, 3.33% and 3.30% respectively.

The combined, aligned data matrix for *rps16–trnQ*, *trnC–rpoB*, *trnD–trnT*, *trnT–trnL* and *ndhF* 3' end was 4,885 bp long, with 49 indels. Only 223 characters (4.6%) were parsimony informative (PIC). A total of 172 sequences were used for this study and the data matrix was composed of 164,997 data points excluding the data scored as missing, which was 6.71% (11,823) of the total data. Table 2 summarizes statistics for each of the 11 regions selected for the temperate species. Relatively low genetic variation was found among the sequences for the chloroplast markers used. Maximum Parsimony analysis of the combined five-region dataset resulted in 1019 shortest trees of 967 steps, with a consistency index (CI) of 0. 6014 (uninformative characters excluded) and a retention index of 0.7352.

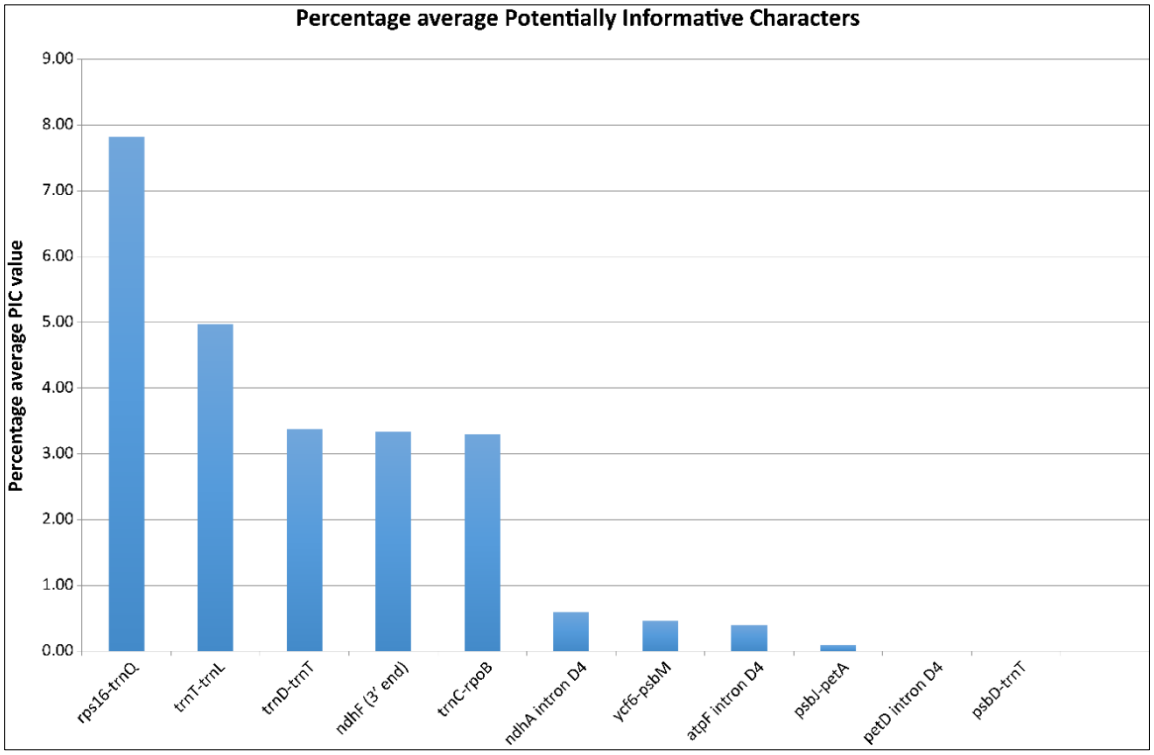

FIGURE 1. Percentage Potentially Informative Character values for all 11 chloroplast regions. For the regions *rps16–trnQ, trnC–rpoB, trnD–trnT, trnT–trnL and ndhF 3'* the PIC values include the outgroups. For the regions *psbD-trnT*, *psbJ-petA* and *ycf6-psbM* the PIC values include only the ingroup species.

TABLE 2. Statistics and evolutionary models for each region and the combined analyses. Evolutionary models and phylogenetic analyses were conducted only for the first five regions *ndhF* (3' end), *rps16-trnQ*, *trnC-rpoB*, *trnD-trnT* and *trnT-trnL*, which showed reasonable PIC values. Statistics for the first five regions are based on the five region, 36 taxon data matrix. * indicates the combined data set of five regions including all the indels. § indicates the markers that showed very low genetic variation for the temperate clade and amplified for a subset of species (~7 species). bp = base pairs, CI = Consistency Index, excluding informative characters, MP = Maximum Parsimony, PIC = Parsimony Informative Characters, RI = Retention Index. Models are based on the Akaike information criterion (AIC) calculations implemented in JmodelTest 0.1.

Partition	Length (bp)	Indels	Total char.	Char., no gaps	PIC	MP trees	MP length	CI	RI	Model
ndhF (3' end)	1,170	0	1,170	1,120	39	195	174	0.6324	0.6753	TVM+G
rps16-trnQ	1,740	14	1,754	1,140	136	193	235	0.6383	0.7862	GTR+G
trnC-rpoB	1,395	14	1,409	965	46	174	191	0.6533	0.8452	TVM+I+G
trnD-trnT	1,335	13	1,348	960	45	194	193	0.6883	0.7838	TVM+G
trnT-trnL	925	8	933	650	46	168	170	0.6118	0.7381	GTR+I
5-region, all data*	6,565	49	6,614	4,835	223	1019	967	0.6014	0.7352	TVM+G
§*atpF* intron D4	755	2	757	---	3	---	---	---	---	---
§*ndhA* intron D4	999	1	1,000	---	6	---	---	---	---	---
§*petD* intron D4	765	0	765	---	0	---	---	---	---	---
§*psbD-trnT*	1,500	0	1,500	---	0	---	---	---	---	---
§*psbJ-petA*	1,020	0	1,020	---	1	---	---	---	---	---
§*ycf6-psbM*	870	0	870	---	4	---	---	---	---	---

As shown in Figure 2, MP, ML and BI analyses of the combined, 5-region dataset all recovered 11 major temperate bamboo lineages: *Bergbambos* (Clade I), African alpine bamboos (Clade II), *Chimonocalamus* J.R. Xue & T. P. Yi (1979: 76) (Clade III), *Shibataea* Makino ex Nakai (Makino1912: 236) clade (Clade IV), *Phyllostachys* Siebold & Zuccarini (Muroi 1963: 13) clade (Clade V), *Arundinaria* clade (Clade VI), *Thamnocalamus* (Clade VII), *Indocalamus wilsonii* (Rendle 1914: 63) Chao & Chu (1981: 43) (Clade VIII), *Gaoligongshania* D.-Z. Li, C.-J. Hsueh & N.-H. Xia (1995: 598) (Clade IX), *Indocalamus sinicus* (Hance 1876: 336) Nakai (1925: 148) (Clade X) (Triplett & Clark 2010, Zeng *et al*. 2010), and the Sri Lankan *Arundinaria* clade (Clade XII). In the current study, the monophyly of the temperate woody bamboo clade was highly supported, with 100% Maximum Parsimony Bootstrap, 100% Maximum Likelihood Bootstrap and 1.00 PP. The Sri Lankan *Arundinaria* clade received maximal support (Maximum Parsimony Bootstrap 100%; Maximum Likelihood Bootstrap 100%; PP 1.0). Relationships within the Sri Lankan *Arundinaria* clade were unresolved, as was the case for the other major lineages, except for the *Arundinaria* clade. Further, the African alpine bamboo (Clade II), *Shibataea* (Clade IV) and *Arundinaria* (Clade VI) clades each received moderate MP support, but strong support from the BI (all 1.00 PP). Only the African alpine bamboos and the *Arundinaria* clades received strong support from the ML analysis (95% and 95% respectively) whereas the *Shibataea* clade received no MLBS support. In addition, five lineages were represented by a single species: *Bergbambos tessellata* (Nees von Esenbeck 1834: 482) Stapleton (2013: 99), *Indocalamus wilsonii*, *Thamnocalamus spathiflorus* (Trin.) Munro (1868: 34),

Gaoligongshania megalothyrsa (Handel-Mazzetti 1936: 1271) D.Z. Li, J.R. Xue & N.H. Xia (Li, Hsueh & Xia 1995: 601) and *Indocalamus sinicus*. However, *Chimonocalamus pallens* J.R. Xue & T.P. Yi (1979: 78) (the type species of *Chimonocalamus*) did not cluster with *Chimonocalamus montanus* J.R. Xue & T.P. Yi (1979:80) and thus *Chimonocalamus* was resolved as polyphyletic.

Results of the K-H test are summarized in Table 3. Based on the K-H test, our data reject the monophyly of a group consisting of Sri Lankan *Arundinaria*, *Arundinaria* s.s. (Clade VI), *Thamnocalamus* (Clade VII), *Bergbambos tessellata* (Clade I), and the African alpine bamboos (Clade II), i.e., the hypothesis that the Sri Lankan *Arundinaria* species belong to *Arundinaria* in the broad sense. Data also reject the monophyly of Sri Lankan *Arundinaria* + *Arundinaria* s.s. and monophyly of Sri Lankan *Arundinaria* + the *Thamnocalamus* clade. Further, monophyly of the Sri Lankan *Arundinaria* + African alpine bamboos is rejected by the K-H test. Despite the lack of resolution among lineages, the K-H test could not reject the monophyly of the Sri Lankan *Arundinaria* species (Clade XII) plus the South African mountain bamboo *Bergbambos tessellata*.

TABLE 3. Hypotheses regarding clades and relationships among them. All hypotheses were tested under MP using the Kishino-Hasegawa test. The difference between the MP trees and those consistent with the constraint were reported. * indicates $p < 0.05$.

Hypothesis	**Results of K-H Test**
Sri Lankan *Arundinaria*, *Arundinaria* s.s, *Thamnocalamus*, *Bergbambos*, and African Alpine bamboos are monophyletic	Reject (+7 steps, p=0.000*)
Sri Lankan *Arundinaria* and *Arundinaria* s.s are monophyletic	Reject (+8 steps, p=0.000*)
Sri Lankan *Arundinaria* and *Thamnocalamus* are monophyletic	Reject (+7 steps, p=0.000*)
Sri Lankan *Arundinaria* and *Bergbambos* are monophyletic	Cannot reject (-2 steps, p=0.078)
Sri Lankan *Arundinaria* and African Alpine bamboos are monophyletic	Reject (+1 step, p=0.000*)

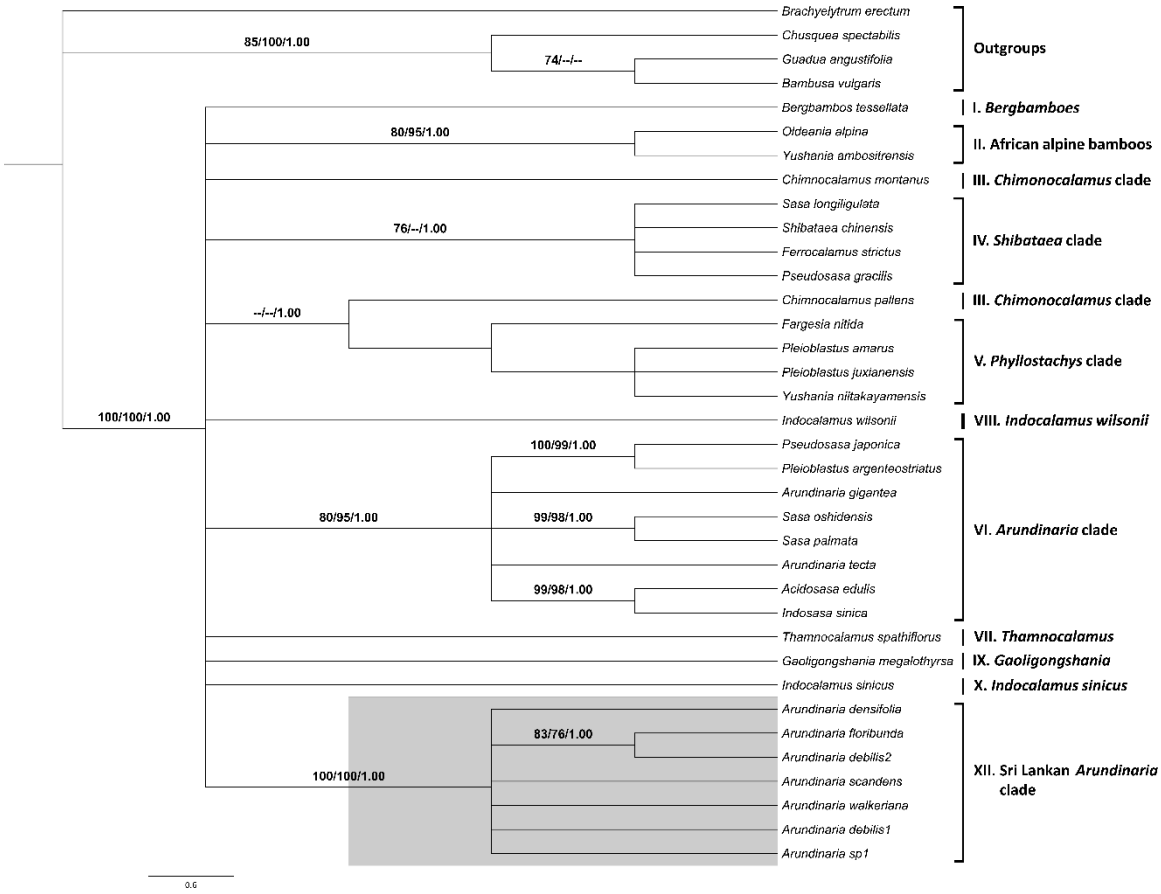

FIGURE 2. Strict consensus of 1019 most parsimonious trees based on the five-region cpDNA dataset (*rps16–trnQ, trnC–rpoB, trnD–trnT, trnT–trnL, ndhF 3'*). Shaded region indicates the well supported Sri Lankan *Arundinaria* clade. Numbers indicate bootstrap values ≥ 70% from MP and ML analyses and posterior probabilities ≥ 0.95 from the BI analyses, respectively. Note that Clade XI is not shown in the tree because it was unsampled.

Results of the morphological comparison are reported in Table 4. Some of the characters, such as culm leaf auricles and palea apex (Figure 3), were variable within the Sri Lanka *Arundinaria* clade. Culm leaf blade position was quite variable within the Sri Lankan *Arundinaria*, *Arundinaria* s.s., *Thamnocalamus spathiflorus* and *Yushania* clades, whereas other characters listed in Table 4 were much more consistent.

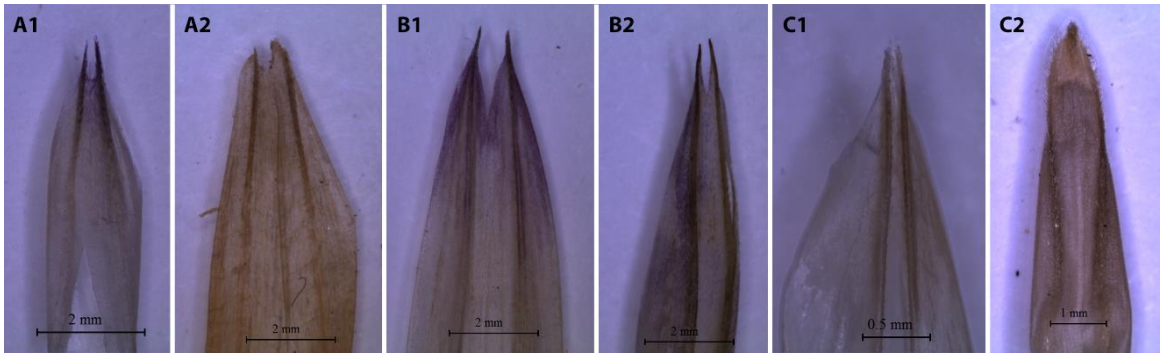

FIGURE 3. The three different types of palea apices. **A1**—biapiculate (sinus shallow) palea apex of *Yushania niitakayamensis* (Hayata) P.-C. Keng (1957: 357) and **A2**—biapiculate (sinus shallow) palea apex of *Bergbambos tessellata*; **B1**—long-divided tips (sinus deep) palea of *Arundinaria gigantea* and **B2**—long-divided tips (sinus deep) palea of *Fargesia spathacea* Franchet (1893: 1067); **C1**—acute, undivided palea apex of *A. debilis* and **C2**—acute, undivided palea apex of *Oldeania alpina* (K. Schum.) Stapleton (2013: 100).

Discussion

As predicted, the chloroplast genome appears to be conserved within the temperate clade, consistent with prior studies (Triplett *et al*. 2010, Zeng *et al*. 2010). Even though 11 relatively variable markers were tested in the current study, only five markers were selected for further phylogenetic analyses due to the lack of molecular variation within six of the markers as seen in a subset of seven species from across the temperate woody bamboo clade. These five markers, *ndhF* (3' end), *rps16-trnQ*, *trnC-rpoB*, *trnD-trnT* and *trnT-trnL*, were successfully utilized in previous studies to understand the relationships among Arundinarieae (Triplett & Clark 2010, Zeng *et al*. 2010, Yang *et al*. 2013).

The six markers that did not provide enough molecular variation for further use in this study had not been previously utilized in the temperate woody bamboo clade. Watts *et al*. (2008) successfully amplified and sequenced the markers *petD*, *atpF* and *ndhA* from domain IV (D4) of chloroplast group II introns, on a sample of closely related

species in *Chusquea* Kunth (1822: 151), a genus of Neotropical woody bamboos. Shaw *et al*. (2007) utilized *psbD-trnT, psbJ-petA* and *ycf6-psbM* in a wide sample of flowering plants, both eudicots and monocots (including Poaceae) and revealed that these markers could be potential candidates to resolve genus and species-level molecular phylogenetic questions. However, none of these six markers showed enough variation in the temperate woody bamboo clade to resolve phylogenetic relationships due to their relatively low level of sequence divergence.

Lineages within the Arundinarieae

The current study is the third analysis sampling almost all of the known temperate bamboo clades (Triplett & Clark 2010, Zeng et al. 2010) and the first to include the Sri Lankan *Arundinaria* species. Even though the monophyly of the temperate clade is always highly supported, relationships within the clade have been difficult to resolve based on cpDNA markers alone or cpDNA plus one or two nuclear markers (Triplett & Clark 2010, Zeng *et al*. 2010, Yang *et al*. 2013). Our results, based on five cpDNA markers, are consistent with these previous findings, but the molecular topology we obtained revealed a new, robustly supported major temperate bamboo lineage, designated as Clade XII, that includes all the Sri Lankan species previously recognized as *Arundinaria* by Soderstrom & Ellis (1988).

We were unable to resolve relationships among the 11 lineages of temperate woody bamboos sampled here, consistent with other analyses (Triplett & Clark 2010, Zeng *et al*. 2010, Yang *et al*. 2013). Several factors could account for the poor resolution among the lineages of temperate woody bamboos. An obvious explanation is a lack of phylogenetic signal, reflecting the relatively conserved nature of the plastid genome in

these bamboos (Triplett & Clark 2010). The long generation times (up to 120 years) characteristic of many temperate woody bamboos may also affect the rate of molecular evolution in this group (Janzen 1976, Gaut *et al*. 1997, Smith & Donoghue 2008), although more study is needed to explore this in woody bamboos generally. Triplett *et al*. (2010) also suggested that reticulate evolution is much more significant in the temperate bamboos than previously predicted, but Triplett & Clark (2010) noted that incomplete lineage sorting could be an additional factor. Further, some recent studies have proposed that relatively recent and rapid diversification within Arundinarieae could be another cause for poorly resolved phylogenetic patterns, especially at the generic level (Stapleton *et al*. 2009, Hodkinson *et al.* 2010, Stapleton 2013).

TABLE 4. Comparative table of morphological characters for the "*Arundinaria*" groups. "?" indicates unknown material.

	Sri Lankan *Kuruna* clade [Clade XII]	***Arundinaria* s.s. [Clade VI]**	***Bergbambos tessellata* [Clade I]**	***Thamnocalamus spathiflorus* [Clade VII]**	***Yushania* [Clade V]**	***Fargesia* (*F. spathacea*-type) [Clade V]**	***Indocalamus* (*I. sinicus*-type) [Clade X]**	***Oldeania alpine* [Clade II]**
Vegetative Characters								
Rhizomes	Pachymorph culm bases with short necks	Leptomorph rhizomes	Pachymorph culm bases with short necks (25–30 cm)	Pachymorph culm bases with short necks	Pachymorph culm bases often with long necks	Pachymorph culm bases with short necks	Leptomorph rhizomes	Pachymorph culm bases with long necks
Clump form	Unicaespitose	Unicaespitose or pluricaespitose	Unicaespitose	Unicaespitose	Pluricaespitose	Unicaespitose	Pluricaespitose	Culms solitary
Culm grooves	Absent	Present in *A. gigantea*	Absent	Absent	Absent	Absent	Absent	Present (sulcate)
Supranodal ridge diameter	Prominent, wider than nodes (*A. debilis* & *A. floribunda*) or more or less the same diameter as the adjacent internodes (other 4 spp.)	More or less the same diameter as the adjacent internodes	More or less the same diameter as the adjacent internodes	More or less the same diameter as the adjacent internodes	More or less the same diameter as the adjacent internodes	More or less the same diameter as the adjacent internodes	More or less the same diameter as the adjacent internodes	Well developed, its diameter greater than the adjacent internodes
Culm leaf girdle	Present as a band at least 1mm wide	Absent or poorly developed	Absent or poorly developed	Present as a band at least 1mm wide	Present as a band at least 1mm wide	Absent or poorly developed	Present and usually yellow brown	Present as a band at least 1mm wide
Culm leaf blade position	Reflexed (except erect in *A. densifolia*)	Erect or becoming reflexed	Reflexed	Erect to slightly spreading	Erect to slightly spreading	Reflexed	Reflexed	Usually reflexed

TABLE 4. continued.

	Sri Lankan *Kuruna* clade [Clade XII]	***Arundinaria* s.s. [Clade VI]**	***Bergbambos tessellata* [Clade I]**	***Thamnocalamus spathiflorus* [Clade VII]**	***Yushania* [Clade V]**	***Fargesia* (*F. spathacea*-type) [Clade V]**	***Indocalamus* (*I. sinicus*-type) [Clade X]**	***Oldeania alpine* [Clade II]**
Foliage leaf sheath auricles/ fimbriae	Fimbriae present	Fimbriae present (except *A. tecta* with fimbriate auricles)	Small fimbriate auricles present	Well-developed fimbriate auricles present	Fimbriae present	Fimbriae present	?	Fimbriate auricles present
Foliage leaf sheath	Strongly keeled at least near the summit	Rounded on the back	Rounded on the back	Strongly keeled at least near the summit	Strongly keeled at least near the summit	Narrowly rounded to strongly keeled at least near the summit	Rounded on the back	Strongly keeled at least near the summit
Foliage leaf sheath persistence	Persistent	Persistent	Persistent	Deciduous	Persistent	Persistent	?	Deciduous
Number of shortened internodes at the base of the branch complement	2–9	0–1 in *A. gigantea*; 2– several in *A. tecta* and *A. applachiana*	4–5	2–3	3–5	2–9	2–5	2–3
Node	Without roots	Without roots	Without roots	Without roots	Without roots	Without roots	Without roots	With ring of thorn like roots at the nodes
Branch sheathing	Complete	Complete	Reduced	Complete	Reduced	Reduced	?	Reduced
Branch orientation	Initially erect, then spreading	Erect to spreading	Erect	Erect to slightly spreading	Erect to spreading	Erect	?	Spreading
Secondary branch initiation area	2nd or 3rd compressed internode	2nd, 3rd or 4th compressed internode	4th compressed internode	2nd compressed internode	2nd or 3rd compressed internode	2nd or 3rd compressed internode	?	2nd compressed internode

TABLE 4. continued.

	Sri Lankan *Kuruna* clade [Clade XII]	***Arundinaria* s.s. [Clade VI]**	***Bergbambos tessellata* [Clade I]**	***Thamnocalamus spathiflorus* [Clade VII]**	***Yushania* [Clade V]**	***Fargesia* (*F. spathacea*-type) [Clade V]**	***Indocalamus* (*I. sinicus*-type) [Clade X]**	***Oldeania alpine* [Clade II]**
Reproductive Characters								
Synflorescence type	Open racemes or panicles, not unilateral	Open racemes or panicles, not unilateral	Contracted raceme, not unilateral	Contracted panicle, not unilateral	Open panicle, not unilateral	Densely contracted unilateral raceme	Raceme or an open panicle, not unilateral	Panicle, not unilateral
No. of fertile florets per spikelet	2–6 (except *A. densifolia*, with one)	7–12	1–3	2–7	1–7	3–4	3–4	2–11
Presence of spatheate empty bracts at the base of the synflorescence	Absent	Absent	One or more present	One well-developed spatheate empty bract present	One or sometimes none	Two present	Absent	Absent
Presence of subtending bracts	Absent	Absent	Present at each node, well developed with a sheath and a blade	Present at each node, variable in size and development, but some well developed	Present as a very small bract only at the first node, otherwise absent	Present at each node except the terminal node, variable in size	Usually very small bracts present	Absent
Palea apex	Biapiculate (sinus shallow) (*A. floribunda* & *A. densifolia*) or acute, undivided (other 3spp.)	Tips long-divided (sinus deep)	Biapiculate (sinus shallow)	Biapiculate (sinus shallow)	Biapiculate (sinus shallow)	Tips long-divided (sinus deep)	Biapiculate (sinus shallow)	Acute, undivided

As noted previously, Clade XI (consisting of the single species *Ampelocalamus calcareus*) was not included in this analysis due to its recent discovery (Yang *et al.* 2013) and the lack of material for comparable sequencing.

Hypothesis testing

The K-H test could not reject the possibility of the monophyly of the Sri Lankan *Arundinaria* species (Clade XII) plus the South African mountain bamboo *Bergbambos tessellata*, although morphological characters clearly differentiate Sri Lankan *Arundinaria* species from *Bergbambos tessellata* (Table 4), suggesting that these two clades are distinct. Linder *et al.* (1997) proposed an Indian – Sri Lankan – Madagascan – Southern African biogeographical pattern for *Crinipes* Hochstetter (Fürarohr 1855: 279) (Poaceae: Arundinoideae). The possible monophyly of Sri Lankan *Arundinaria* species (Clade XII) and *Bergbambos tessellata* revealed by the K-H test of the current study suggests the possibility of a similar biogeographical pattern in this group, but additional sampling of Indian and Madagascan *Arundinaria* species as well as better resolution among temperate bamboo lineages are both needed before this question can be addressed.

Generic status of the Sri Lankan Arundinaria clade

The morphological comparison eliminated the possibility that any of these Sri Lankan *Arundinaria* species or other "*Arundinaria*" groups should be classified within *Fargesia*, *Indocalamus*, *Chimonobambusa* or *Chimonocalamus*. *Fargesia* has a densely contracted unilateral raceme with long divided (deep sinus) palea apices that separates it from the Sri Lankan *Arundinaria, Thamnocalamus, Bergbambos tessellata*, African alpine bamboos and *Yushania*. In addition, both *Indocalamus* and *Chimonobambusa* have leptomorph rhizomes while all the Sri Lankan *Arundinaria, Thamnocalamus*,

Bergbambos tessellata, African alpine bamboo and *Yushania* clades have pachymorph culm bases (= pachymorph rhizomes as usually described in bamboo literature). Further, the presence of basally grooved culms and synflorescences with pseudospikelets distinguish *Chimonobambusa* from *Arundinaria* s.s., Sri Lankan *Arundinaria*, *Thamnocalamus*, *Bergbambos tessellata*, African alpine bamboos and *Yushania*. Both *Chimonobambusa* and *Chimonocalamus* have subequal multiple (or apparently multiple) buds per node which also provide evidence that these two genera are distinct from the *Arundinaria* s.s., Sri Lankan *Arundinaria*, *Thamnocalamus*, *Bergbambos tessellata*, African alpine bamboo and *Yushania* clades.

Unlike the K-H test, which was unable to reject the monophyly of the Sri Lankan *Arundinaria* clade + *Bergbambos tessellata*, the morphological characters indicate consistent differences. Both vegetative and reproductive morphological characters clearly differentiate the Sri Lankan *Arundinaria* species from *Bergbambos tessellata*. The presence of usually hispid culm leaves and the general absence of both culm leaf auricles and foliage leaf fimbriate auricles separate the Sri Lankan *Arundinaria* clade from *Bergbambos tessellata*, which has glabrous culm leaves and fimbriate auricles in both culm and foliage leaves. As discussed in Stapleton (2013), branch sheathing is an important character that could be used to differentiate these clades. Compared to *Bergbambos tessellata*, the Sri Lankan *Arundinaria* clade has a complete set of sheaths at the nodes (complete sheaths), while *Bergbambos tessellata* has lost some of the sheaths at the nodes (reduced sheaths). Even though both of these clades possess non-unilateral synflorescences, the Sri Lankan *Arundinaria* species have open racemes or panicles, but *Bergbambos tessellata* has contracted racemes. In addition, the presence of one or more

spatheate bracts at the base of the synflorescence and subtending bracts at the synflorescence nodes in *Bergbambos tessellata* further supports the difference between these two clades, as these structures are completely absent in the Sri Lankan clade. Finally, the shape of the palea apex (Figure 3) is one of the best characters to differentiate among these temperate woody bamboo clades, particularly between the Sri Lankan clade (acute, undivided tips) and *Bergbambos* (biapiculate with a shallow sinus). We note that Stapleton (2013) reported a single fertile floret per spikelet in *Bergbambos tessellata*, which could serve as an additional distinction between *Bergbambos* and the Sri Lankan clade. However, based on our observations and the morphological analysis of Soderstrom & Ellis (1982), *Bergbambos tessellata* has one to three fertile florets plus at least one apical reduced or rudimentary floret, so floret number is not a useful character in this case. But overall, the molecular evidence combined with the morphology supports the recognition of a new genus that will accommodate all the Sri Lankan temperate woody bamboo species, which we here describe.

Although we were unable to examine any material of *Ampelocalamus calcareus* (Clade XI) for the morphological analysis, the strongly arching to hanging culms of this species, along with the well-developed, fimbriate auricles on its culm leaves and foliage leaves (Yi *et al.* 2008), mean it is unlikely to have a close relationship to the Sri Lankan *Arundinaria* clade. We therefore exclude it from further consideration.

According to the molecular analyses and morphological comparison all the reported native Sri Lankan *Arundinaria* species, namely *Arundinaria debilis*, *A. densifolia*, *A. floribunda*, *A. scandens* and *A. walkeriana*, belong to this new Sri Lanka temperate woody bamboo genus. However, based on morphology, especially of the

spikelets, previous studies suggest a possible relationship between *Arundinaria densifolia* and allies in Sri Lanka and South India (Campbell, unpubl.). Seethalakshmi & Muktesh Kumar (1998) described *A. floribunda* and *A. walkeriana* as being distributed in both South India and Sri Lanka in very similar habitats. These taxa of "*Arundinaria*" from India were not sampled for the current analysis, so we could not confirm their identification. Furthermore, *A. wightiana* Nees von Esenbeck (1834: 482) is also reported to occur in both South India and Sri Lanka (Seethalakshmi & Muktesh Kumar 1998), but in our field work this species could not be located in Sri Lanka. Further, no previous studies relating to the Flora of Ceylon (Soderstrom & Ellis 1988, Dassanayake & Fosberg 1994) indicated any distribution of *A. wightiana* in Sri Lanka. Morphological comparison of *A. wightiana* and the Sri Lankan *Arundinaria* species shows some resemblance of the Indian taxon mainly with *A. debilis* and *A. floribunda* from Sri Lanka. Presence of a well-developed supranodal ridge, abaxially hispid culm leaves, fimbriate culm leaf sheath summits and foliage leaves with fimbriae are the main similarities among these three species. However, the very small culm leaf blade, relatively long fimbriae on both culm leaf sheath summits and on the foliage leaf auricles distinguish *A. wightiana* from the Sri Lankan *Arundinaria* species. In addition, the abaxial culm leaf surfaces of *A. wightiana* are covered by dark brown irritating hairs, while the abaxial culm leaf surfaces of Sri Lankan *Arundinaria* species are covered with non-irritating whitish brown hairs. Thus, morphological comparison suggests that *A. wightiana* could belong to the Sri Lankan *Arundinaria* clade, but this species needs further study and it has not yet been sampled in a molecular analysis. In addition, Dransfield (2003) reported six *Arundinaria* species endemic to Madagascar, but only one (*Yushania ambositrensis*) has been sampled in this

and previous molecular analyses (Triplett & Clark 2010), and it clusters with *Oldeania alpina*. Stapleton (2013) suggests that temperate bamboos radiated from India to Asia, Africa, and North America. Therefore, it is possible that one or more of these Madagascan *Arundinaria* species will ultimately be shown to belong to the Sri Lankan *Arundinaria* clade, but these species are poorly known and more work is needed before they can be assigned to an appropriate genus.

Conclusions

A major finding of this investigation is the resolution of a robustly supported twelfth lineage, the Sri Lankan *Arundinaria* clade (Clade XII), within the temperate woody bamboos (Arundinarieae). Although the position of this clade with respect to the other ten sampled clades of temperate woody bamboos is unresolved, alternate hypothesis testing rejects monophyly of the Sri Lankan *Arundinaria* in combination with *Arundinaria* s.s. (restricted to North America) or other clades considered as *Arundinaria* in the broad sense, with the exception of *Bergbambos tessellata* (Clade I) from South Africa. A morphological analysis of these clades, however, provides characters to distinguish the Sri Lankan *Arundinaria* clade from *Bergbambos*.

The current study therefore provides robust molecular and morphological support for the recognition of the Sri Lankan *Arundinaria* clade as a new genus, here named *Kuruna*. We also make new combinations in *Kuruna* for the five described species in Sri Lanka; a complete taxonomic revision for this group is currently in preparation. We emphasize that the south Indian species of *Arundinaria* have not yet been sampled in a molecular study, and therefore concepts of *Bergbambos*, *Kuruna*, African alpine

bamboos or other temperate woody genera may ultimately change, and we also cannot rule out the recognition of additional lineages within the temperate woody bamboos.

We suggest the use of other markers such as low copy nuclear markers and plastid genome sequences to better understand phylogenetic relationships within the highly complex, taxonomically difficult temperate woody bamboo clade. Though the topology that we obtained was imperfectly resolved, it can still serve as a foundation for testing biological or biogeographic hypotheses. The integration of more polymorphic markers into molecular analyses is necessary to obtain better resolution in order to critically examine divergence times, biogeography and morphological evolution within temperate woody bamboos.

Taxonomic Treatment

Kuruna* Attigala, Kaththriarachchi & L. G. Clark, *gen. nov.

TYPE: *Arundinaria debilis* Thwaites (1864: 375).

Kuruna debilis (Thwaites) Attigala, Kaththriarachchi & L. G. Clark

Diagnosis:—Differs from *Arundinaria* s.s. by its pachymorph culm bases with short necks, culm leaves usually abaxially hispid, culm leaf girdles ca. 1 mm wide, culm leaf auricles absent and the palea apex biapiculate to acute. The following characters differentiate *Kuruna* from other "*Arundinaria*" groups (*Bergbambos,* African alpine bamboos, *Thamnocalamus* and *Yushania)*: pachymorph culm bases with short necks, unicaespitose clumps, culm leaf girdles present as a band ca. 1 mm wide, usually abaxially hispid culm leaves with non-irritating hairs, persistent foliage leaf sheaths and complete branch sheathing, palea apex biapiculate (sinus shallow) to acute and

undivided, and both spatheate empty bracts at the base of the synflorescence and subtending bracts absent.

Description:—*Culm bases* pachymorph, short necked, two or more tillers per culm base present. *Culms* woody, erect, shrubby or scandent; midculm internodes usually hollow, terete to flattened or shallowly sulcate above the branches, smooth, wall thickness (ratio of 2 times wall thickness: culm diameter) moderate (ratio 0.31–0.45) to thick (ratio 0.46–0.61), lacuna greater than 1/3 of the diameter of the culm; nodal lines horizontal; supranodal ridge mostly conspicuous. *Culm leaves* clearly differentiated from the foliage leaves; girdle present as a band at least 1mm wide; sheath usually abaxially hispid with non-irritating hairs, sheath apex usually symmetrically concave, fimbriate, sheath summit extension present; blade usually reflexed, sessile, more or less narrowly triangular. *Branching pattern* intravaginal. *Branch complement* derived from one bud per node and born on a promontory; bud prophyll margins unitary, free; two to several compressed proximal internodes at the base of the primary axis; secondary branches subequal to the primary axis, developing from the second or third compressed internodes and above; central primary branch smaller in diameter than the main culm; three or more leaves per leafy branch and leaf branch apex growth indeterminate. *Foliage leaves* fimbriate, erect, all leaves with a sheath and a blade; sheath usually strongly keeled at least near the summit. *Synflorescences* paniculate or racemose, with 1–2 orders of branching, open, terminating the leafy branches, both spatheate bracts at the base and subtending bracts absent. *Spikelets* pedicellate, laterally compressed, consisting of two glumes, usually two to six female-fertile florets per spikelet (but *A. densifolia* only one); rachilla extension hairy, bearing a rudimentary floret, shorter than or equal to about half the length of a

fertile floret; glumes shorter than the spikelet, unawned; lemmas unawned; paleas 2-keeled, the keels winged, apex biapiculate or acute, sulcus well developed for the full length. Lodicules 3, ciliate; stamens 3, filaments free, anther apex lobes rounded, anther connective lower than the apical anther lobes; stigmas 2 or 3, plumose. Fruit a basic caryopsis with a linear hilum as long as the fruit.

Etymology:—The generic name *Kuruna* is derived from the common name in Sinhalese of the native Sri Lankan *Arundinaria* group, "KuruUna". "Kuru" means dwarf, and "Una" means bamboo.

Distribution:—Warm temperate and montane regions (both forests and open grasslands) of Sri Lanka (Soderstrom & Ellis, 1988).

Following are the new combinations for all the native *Arundinaria* species in Sri Lanka:

1. *Kuruna debilis* (Thwaites) Attigala, Kaththriarachchi & L. G. Clark, *comb. nov*.

Basionym:—*Arundinaria debilis* Thwaites (1864: 375).

2. *Kuruna densifolia* (Munro) Attigala, Kaththriarachchi & L. G. Clark, *comb. nov*.

Basionym:—*Arundinaria densifolia* Munro (1868: 32).

3. *Kuruna floribunda* (Thwaites) Attigala, Kaththriarachchi & L. G. Clark, *comb. nov*.

Basionym:—*Arundinaria floribunda* Thwaites (1864: 375).

4. *Kuruna scandens* (Soderstrom & Ellis) Attigala, Kaththriarachchi & L. G. Clark, *comb. nov*.

Basionym:—*Arundinaria scandens* Soderstrom & Ellis (1988: 20).

5. *Kuruna walkeriana* (Munro) Attigala, Kaththriarachchi & L. G. Clark, *comb. nov*.

Basionym:—*Arundinaria walkeriana* Munro (1868: 21).

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APPENDIX. ALPHABETICAL LIST OF VOUCHER SPECIMENS UTILIZED IN THE CPDNA ANALYSIS

All the vouchers are deposited at ISC unless otherwise indicated.

Taxon	**Voucher**	**Source**	**GenBank accession no.**				
			ndhF (3' end)	***rps16–trnQ***	***trnC–rpoB***	***trnD–trnT***	***trnT-trnL***
Acidosasa edulis	Triplett148	GenBank	---	FJ643789	FJ643882	FJ643975	FJ644126
*Arundinaria debilis*1	Attigala123	Horton Plains, Sri Lanka	KJ638186	KJ638172	KJ638200	KJ638191	KJ638179
*A. debilis*2	Attigala147	Hayes-Gongala, Sri Lanka	---	KJ638173	KJ638201	KJ638192	KJ638180
A. densifolia	Attigala126	Horton Plains, Sri Lanka	KJ638187	KJ638174	KJ638197	KJ638193	KJ638181
A. floribunda	Attigala139	Amanawala-Ampane, Sri Lanka	---	KJ638175	---	---	KJ638182
A. gigantea	Triplett 197	GenBank	FJ643707	FJ643794	FJ643887	FJ643980	FJ644131
A. scandens	Attigala166	Pidurutalagala summit, Sri Lanka	KJ638188	KJ638176	KJ638202	KJ638194	KJ638183
A. tecta	Triplett 173	GenBank	FJ643708	FJ643795	FJ643888	FJ643981	FJ644132
A. walkeriana	Attigala162	Adam's Peak, Sri Lanka	KJ638189	KJ638177	KJ638198	KJ638195	KJ638184
Arundinaria sp1	Attigala146	Hayes-Gongala, Sri Lanka	KJ638190	KJ638178	KJ638199	KJ638196	KJ638185
Bambusa vulgaris	Sánchez-Ken 666	GenBank	FJ643709	FJ643796	FJ643889	FJ643982	FJ644133
Brachyelytrum erectum	Triplett 199b	GenBank	U22005	FJ643799	FJ643892	FJ643985	FJ644136
Chimnocalamus montanus	Triplett 261	GenBank	---	FJ643807	FJ643900	FJ643993	FJ644144
Chimnocalamus pallens	Triplett 238	GenBank	FJ643712	FJ643808	FJ643901	FJ643994	FJ644145
Chusquea spectabilis	Clark 919	GenBank	AF182355	FJ751698	FJ751725	FJ751752	---
Fargesia nitida	Triplett 222	GenBank	---	FJ643813	FJ643906	FJ643999	FJ644150
Ferrocalamus strictus	Campbell 10	GenBank	FJ643713	FJ643815	FJ643908	FJ644001	FJ644152
Gaoligongshania megalothyrsa	Xue 9401 (KUN)	GenBank	---	GU354641	GU354481	GU354801	GU354961
Guadua angustifolia	Clark & Londoño 931	GenBank	FJ643714	FJ643817	FJ643910	FJ644003	FJ644154
Indocalamus sinicus	Zeng & Zhang 06081 (KUN)	GenBank	---	GU354673	GU354513	GU354833	GU354993
Indocalamus wilsonii	Zhang 07088	GenBank	---	GU354626	GU354466	GU354786	GU354945
Indosasa sinica	Triplett 267	GenBank	FJ643715	FJ643827	FJ643920	FJ644013	FJ644164
Pleioblastus amarus	Zhang 07082 (KUN)	GenBank	---	FJ643836	FJ643929	FJ644022	FJ644173
Pleioblastus argenteostriatus	Triplett 66	GenBank	---	FJ643837	FJ643930	FJ644023	FJ644174
Pleioblastus juxianensis	Triplett 117	GenBank	---	FJ643841	FJ643934	FJ644027	FJ644178

APPENDIX continued.

Taxon	**Voucher**	**Source**	**GenBank accession no.**				
			ndhF (3' end)	***rps16–trnQ***	***trnC–rpoB***	***trnD–trnT***	***trnT-trnL***
Pseudosasa gracilis	Zhang 06107 (KUN)	GenBank	---	FJ643849	FJ643942	FJ644035	FJ644186
Pseudosasa japonica	Triplett 122	GenBank	FJ643723	FJ643851	FJ643944	FJ644037	FJ644188
Sasa longiligulata	Zeng 061213 (KUN)	GenBank	---	FJ643859	FJ643952	FJ644045	FJ644196
Sasa oshidensis	Triplett 161	GenBank	---	FJ643860	FJ643953	FJ644046	FJ644197
Thamnocalamus spathiflorus	Clark 1319	GenBank	FJ643728	FJ643876	FJ643969	FJ644062	FJ644213
Bergbambos tessellata	Triplett 202	GenBank	FJ643729	FJ643877	FJ643970	FJ644063	FJ644214
Oldeania alpina	Fadenet al. 96/413 (US)	GenBank	FJ643730	FJ643878	FJ643971	FJ644064	FJ644215
Yushania ambositrensis	Dransfield 1353	GenBank	---	FJ643879	FJ643972	FJ644065	FJ644216
Yushania niitakayamensis	March 28	GenBank	---	FJ643881	FJ643974	FJ644067	FJ644218

CHAPTER 3

TAXONOMIC REVISION OF THE TEMPERATE WOODY BAMBOO GENUS *KURUNA* (POACEAE: BAMBUSOIDEAE: ARUNDINARIEAE)

Modified from a paper in press in *Systematic Botany*

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Abstract

Kuruna is distinguished by a combination of vegetative and reproductive characters including features of rhizomes, branching and synflorescences. Here seven species, distributed in Sri Lanka and southern India, are included in this genus: *Kuruna debilis*, *K. densifolia*, *K. floribunda*, *K. scandens*, *K. walkeriana*, ***K. wightiana*** (here transferred to *Kuruna*) and the newly described ***K. serrulata***. This revision includes an updated description of the genus, detailed descriptions for all seven species, line illustrations for all species, and a morphological key for their identification.

Keywords: *Arundinaria*, *Kuruna serrulata*, southern India, Sri Lanka, taxonomy.

The subfamily Bambusoideae (Poaceae) comprises three tribes: tropical woody bamboos (Bambuseae), temperate woody bamboos (Arundinarieae) and herbaceous bamboos (Olyreae). Arundinarieae, which includes approximately 550 species, is strongly supported as monophyletic by a significant amount of molecular evidence (Bamboo Phylogeny Group [BPG] 2012; Kelchner et al. 2013; Attigala et al. 2014).

Arundinaria Michaux is the oldest generic name within the tribe and over 400 species have at one time or another been classified within it. But previous studies revealed that *Arundinaria* s.s. is composed of only three species from North America: *A. gigantea* (Walter) Muhl. (type), *A. tecta* (Walter) Muhl. and *A. applachiana* Triplett, Weakley & L. G. Clark (Triplett and Clark 2010). Thus, a taxonomic revision is needed for the temperate woody bamboo species traditionally considered as *Arundinaria*, especially those from south Asia, Africa and Madagascar. In a recent study based on plastid DNA sequence data we recognized the Sri Lankan temperate woody bamboos (Clade XII) as the new genus *Kuruna* Attigala, Kathriarachchi & L. G. Clark and made new combinations to accommodate the five known *Arundinaria* species belonging to this clade (Attigala et al. 2014) (Fig. 1).

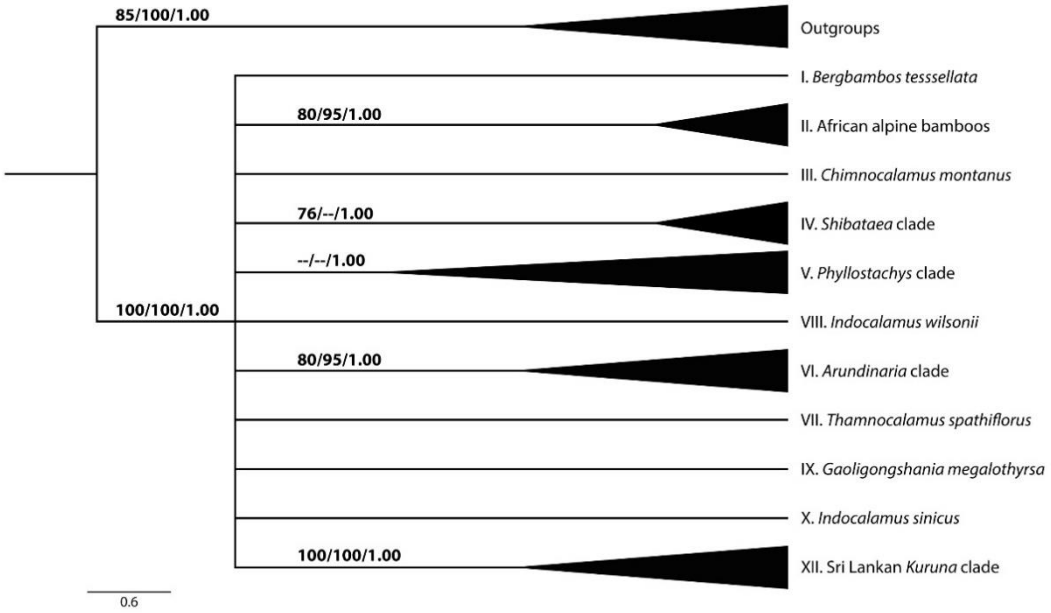


FIG. 1. Summarized strict consensus tree redrawn from Attigala et al. (2014), based on five plastid regions (*rps16–trnQ, trnC–rpoB, trnD–trnT, trnT–trnL, ndhF 3'*). Numbers indicate bootstrap values ≥ 70% from Maximum Parsimony and Maximum Likelihood analyses and posterior probabilities ≥ 0.95 from the Bayesian Inference analyses, respectively. Triangles indicate only where multiple species were sampled per clade and are not proportional to the number of species found in each clade. Note— Clade XI (consisting of the single species *Ampelocalamus calcareus*) was not included in Attigala et al. (2014) due to its recent discovery (Yang et al. 2013) and the lack of material for comparable sequencing.

Here we present updated morphological descriptions for *Kuruna* and for all five reported Sri Lankan temperate woody bamboo species (Soderstrom and Ellis 1988) as well as a newly described species from Sri Lanka. This revision also includes the south Indian temperate woody bamboo *Arundinaria wightiana* Nees, which we here transfer to *Kuruna* based on morphological similarities, as this species has not yet been sampled in any molecular phylogenetic analyses. A morphological key to all seven *Kuruna* species is included, all these species are illustrated to show their diagnostic characters and the distributions for all species are mapped (Fig. 2).

Materials and Methods

Field observations and collections of all six Sri Lankan *Kuruna* species were made primarily by LA. Standard collection procedures for bamboos suggested by Soderstrom and Young (1983) were followed and therefore complete specimens were collected including branch complements, culm leaves, culm nodes and internodes. Rhizomes were collected only for the new species due to collection permit restrictions. Authors followed McClure (1973) and Soderstrom and Ellis (1988) for definitions of structures and morphological terminology. The morphological descriptions were based on detailed study of the collected specimens, direct examination of herbarium specimens (ISC, K, MO, PDA, and US) (Thiers 2012) and information from published literature (Soderstrom and Ellis 1988; Seethalakshmi and Muktesh Kumar 1998; Clayton et al. 2006; Muktesh Kumar 2011). The morphological comparison and measurement of the structures were performed using a Leica S6D (Leica Microsystems Inc., Buffalo Grove, Illinois) stereoscopic microscope. Culm leaf sheaths and blades were measured separately. Foliage leaf length was measured from the base of the pseudopetiole to the tip of the blade and leaf width was measured at the widest point. For all measurements for which a mean was reported (e.g., internode length, wall thickness, lacuna size, culm leaf sheath length, foliage leaf blade length, width, etc.), 10 measurements were taken per collection and averaged. The range was reported within parentheses and represents the range of variation within each species for that structure. As a standardized estimation of culm wall thickness, the ratio between two times the culm wall thickness and culm diameter was calculated (BPG 2005). Spikelets were dissected in Pohl's solution (Pohl 1965). Spikelet length was measured from the base of glume I to the very tip of the

longest part of the spikelet. Illustrations of *K. debilis*, *K. densifolia*, *K. floribunda*, *K. scandens* and *K. walkeriana* were taken directly from Soderstrom and Ellis (1988) and additional illustrations were included for diagnostic structures/characters not previously illustrated for these species. Further, new illustrations were made for the newly described species and the south Indian *K. wightiana*.

The previous investigation of the Sri Lankan temperate woody bamboos by Soderstrom and Ellis (1988) is very thorough, but we found that some of the fine details are missing or misrepresented in some of their illustrations. In Figs. 3i and 4a of *K. debilis* and Fig. 7e of *K. floribunda*, the supranodal ridge is not distinctly illustrated. See Fig. 14F and 14A for illustrations of the conspicuous supranodal ridge of *K. debilis* and *K. floribunda*, respectively. In addition, the palea tips of *K. scandens* (Fig. 9i) and *K. walkeriana* (Fig. 12h) are shown as biapiculate, when they are actually acute, and in Fig. 7a of *K. floribunda,* the foliage leaf blade apex shows a distinct constriction despite there being none in living plants or herbarium specimens we observed. These inaccuracies are pointed out in the figure captions.

Each species was given an International Union for Conservation of Nature (IUCN) Red List category according to the criteria established by the IUCN Red List Categories and Criteria at regional and national levels, Version 4.0 (IUCN 2012). This assessment is based on observations by the corresponding author during a field trip in 2010 to Sri Lanka. Nevertheless, we recommend that population surveys be undertaken to support a better extinction risk assessment of all these *Kuruna* species.

Results and Discussion

Distribution and biogeography

All the Sri Lankan species of *Kuruna* occur in upper montane forests and open montane grasslands in the central province of Sri Lanka while the Indian *Kuruna* species are distributed in the mountains of south India, mainly the Western Ghats (Fig. 2). Of the seven *Kuruna* species, two species are shared between Sri Lanka and south India (*K. floribunda* and *K. walkeriana*), three species are endemic to Sri Lanka (*K. densifolia*, *K. scandens* and *K. serrulata*) and one species is endemic to south India (*K. wightiana*). There is not enough information yet to determine if *K. debilis* is endemic to Sri Lanka, because there are unconfirmed reports of this species in south India (Muktesh Kumar 2011). To date, there are no biogeographical studies available for *Kuruna*. Myers et al. (2000) showed that the moist rain forests of the Western Ghats of peninsular India and the rain forests of south-west Sri Lanka together are considered a refugium of the relict biota of the former Indian plate, which was gradually isolated from other continents for a period of over 25 million years in the mid-Paleocene to late Eocene era (60–35 million years). However, Sri Lanka remained in full contact with India until the last major sea level rise 6000 years ago, which separated these two countries by the narrow and shallow Palk Strait (McLoughlin 2001). Linder et al. (1997) proposed an Indian – Sri Lankan – Madagascan – southern Africa biogeographical pattern for *Crinipes* (Poaceae: Arundinoideae). Thus, given the geographic history of Sri Lanka and India, further studies of *Kuruna* in a biogeographic context could reveal similar biogeographical patterns and insights into dispersal events between Sri Lanka and India. But a formal biogeographical analysis awaits a robust phylogeny of Arundinarieae.

Morphology

The major defining morphological characters of all seven *Kuruna* species are compared and summarized in Table 1.

HABIT—The habits exhibited by *Kuruna* species are mainly of two types. *Kuruna debilis* and *K. scandens* are scandent or clambering with sparse culms. The vine-like long primary and secondary branches of *K. debilis* are supported by trees and shrubs and hang from them. *Kuruna scandens* on the other hand starts off with erect culms which then become arching and scandent. However, the other five species are mainly erect and shrubby with densely packed culms.

RHIZOMES— All *Kuruna* species have pachymorph culm bases with short necks (Figs. 4h, 6j and 8i). Among the seven species of *Kuruna*, *K. densifolia* is the only one with rhizomes producing thick primary roots with air canals (Fig. 6l) due to their occurrence in cold, standing water in swampy open grasslands (wet patana) such as the Horton Plains of Sri Lanka. *Bergbambos tessellata* (Nees von Esenbeck) Stapleton is another temperate woody bamboo species from South Africa, which occurs in similar wet swampy habitats, and possesses root air canals (Soderstrom and Ellis 1982). However, the rhizomes of *K. densifolia* do not have air canals like *Arundinaria tecta* and *A. appalachiana*, which grow in wet or swampy habitats in the southeastern U. S. A. (Triplett et al. 2006).

CULMS—The culm internodes are usually hollow in *Kuruna*, with a well-defined cavity indicating that the internodes developed as hollow from the beginning, and exhibit moderately thick to thick walls. However, *K. serrulata* has both hollow and solid culm internodes. Further, a conspicuous supranodal ridge is present in *K. debilis*, *K. floribunda*

and *K. wightiana*, while the other four species lack a conspicuous supranodal ridge. All *Kuruna* species possess unique internode colors and patterns (Table 1). All have light green to dark green culm internodes (except *K. serrulata* is greenish brown and *K. densifolia* maroon) when they are young but turn mainly maroon, yellowish brown or brownish dark green when they are old. Glabrous or hirsute to scaberulous culm internodes are seen in *Kuruna*. *Kuruna floribunda* and *K. scandens* have a ring of purple hairs at the summit of each internode and hirsute internodes with white hairs respectively when they are young, but the internodes become glabrous with age. *Kuruna wightiana* is the only species that retains scaberulous culm internodes throughout its entire life time. Also, both *K. floribunda* and *K. serrulata* have purple black or black specks on their young culm internodes, which is a characteristic feature that can be used to differentiate these two species from the rest.

BRANCHING PATTERN— All *Kuruna* species have intravaginal branch development beginning with a single primary branch per node derived from a single bud per node. In all species, the primary branch produces from its basal nodes two (three) lateral secondary branches (except in *K. scandens* with three to four secondary branches) arising almost simultaneously. However, *K. densifolia*, *K. scandens*, *K. serrulata*, and *K. walkeriana* go on to form dense clusters of tertiary branches due to the rebranching of the secondary branches as they mature.

CULM LEAVES— Deciduous and persistent culm leaves are both found in *Kuruna*. Persistent culm leaves are seen in *K. densifolia, K. scandens* and *K. serrulata* whereas deciduous culm leaves are common in the other four species. The abaxial indument of the culm leaf sheath is also another important character in *Kuruna*. Of the seven *Kuruna*

species, *K. serrulata* and *K. densifolia* are the only species with abaxially glabrous culm leaf sheaths. In contrast, *K. debilis*, *K. floribunda*, *K. scandens*, *K. walkeriana* and *K. wightiana* have abaxially hispid culm leaf sheaths.

SYNFLORESCENCES— Synflorescences are known for all *Kuruna* species except *K. serrulata*. Synflorescences are racemose or paniculate usually with pulvinate branches (except in *K. densifolia*). Also, all of these species have multiple fertile florets per spikelet, except *K. densifolia* which has only one fertile floret per spikelet.

PALEA TIPS— Two types of palea tips, acute or biapiculate, are observed in *Kuruna*. Acute tips are characteristic of *K. debilis*, *K. scandens* and *K. walkeriana* while biapiculate tips are seen in *K. densifolia*, *K. floribunda* and *K. wightiana*.

Phenology

Very little is known about flowering cycles in *Kuruna*, except that there are no reports of gregarious monocarpy. Flowering cycles in woody bamboos are inferred based on a combination of flowering dates as documented by herbarium specimens sorted by location/population, label information describing the extent of flowering and personal observations (i.e., Guerreiro 2013). Due to the sporadic nature of herbarium voucher collections and the lack of label information, clear flowering patterns could not be inferred for any of the six species of *Kuruna* for which flowering material is known and we were unable to confidently determine the actual flowering intervals of these species. However, for four species, we suggest possible flowering patterns though these could change with more information about flowering events. We suggest that *K. wightiana* may be an annual or sporadic bloomer, while *K. floribunda*, *K. scandens* and *K. walkeriana* have relatively long flowering cycles. Unfortunately, we are unable to suggest anything

about flowering behavior for *K. debilis* and *K. densifolia* due to insufficient herbarium records or label data. No flowering material has been collected for *K. serrulata*.

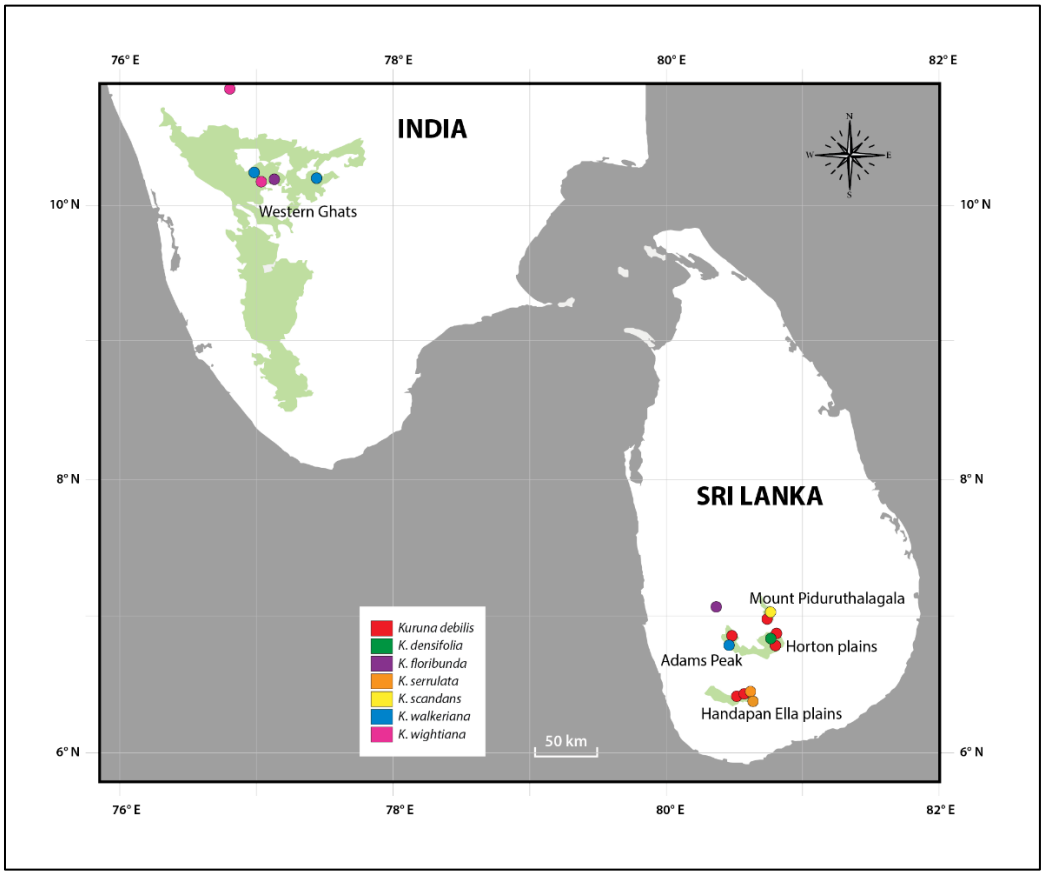


FIG. 2. Distribution of *Kuruna* in Sri Lanka and India.

Taxonomic Treatment

KURUNA Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 199. 2014— *Kuruna debilis* (Thwaites) Attigala, Kathriarachchi & L. G. Clark

Culm bases pachymorph, short-necked, two or more tillers per culm base present. Culms woody, erect, shrubby or scandent, tillering; midculm internodes usually hollow, terete to flattened or shallowly sulcate above the branches, glabrous, all internodes more

or less equally elongated along the culm, ratio of 2 times wall thickness: culm diameter thin to very thick (ratio 0.2–1), lacuna greater than 1/3 of the diameter of the culm; nodes solitary, nodal line horizontal; supranodal ridge mostly inconspicuous or less commonly conspicuous. Culm leaves clearly differentiated from the foliage leaves; girdle present as a band at least 1 mm wide; sheaths usually abaxially hispid, sheath apex usually symmetrically concave, fimbriate, sheath summit extension present; outer ligule absent; blades usually reflexed, sessile, more or less narrowly triangular. Branching pattern intravaginal. Branch complement derived from one bud per node and borne on a promontory; bud prophyll margins unitary, free; primary branch one per node, two to several compressed proximal internodes at its base; primary branch initiation with 2(–3) secondary branches arising almost simultaneously, secondary branches subequal to the primary axis, developing from the second or third compressed internodes and above the primary axis; central primary branch smaller in diameter than the main culm; leaf branch apex growth indeterminate. Foliage leaves per branch complement 3-many, all with a sheath and a blade; sheaths usually strongly keeled at least near the summit, ciliate or fimbriate (with fimbriate auricles in *K. debilis*); blades tessellate, erect, midrib centric. Synflorescences paniculate or racemose, with 1–2 orders of branching, open, terminating the leafy branches, spatheate bracts at the base and subtending bracts both absent. Spikelets purplish, pedicellate, laterally compressed, consisting of two glumes, usually 2–6 female fertile florets per spikelet (but only 1 in *K. densifolia*); rachilla extension hairy, bearing a rudimentary floret, shorter than or equal to about half the length of a fertile floret; glumes shorter than the spikelet, unawned; lemmas unawned; paleas 2-keeled, the keels usually winged, apex biapiculate or acute, sulcus usually well developed for the full

length (except *K. densifolia*). Lodicules 3, more or less subequal, ciliate; stamens 3, filaments free, anther apex lobes rounded, anther connective lower than the apical anther lobes; stigmas 2(–3), plumose. Fruit a basic caryopsis with a linear hilum as long as the fruit.

Etymology

The generic name *Kuruna* is derived from the common name in Sinhalese of the native Sri Lankan *Arundinaria* group, "KuruUna". "Kuru" means dwarf and "Una" means bamboo.

Distribution and Habitat

Kuruna occurs in warm temperate and montane regions (both forests and open grasslands) of Sri Lanka (Soderstrom and Ellis 1988) and the Western Ghats of India (Seethalakshmi and Muktesh Kumar 1998). All *Kuruna* species, except *K. densifolia* and *K. serrulata*, are part of the understory vegetation of montane forests, whereas *K. densifolia* inhabits open montane grasslands and *K. serrulata* occurs in open rocky plains with shallow soil covers. We note that although Muktesh Kumar (2011) has reported *K. debilis*, *K. floribunda*, and *K. walkeriana* as being distributed in south India we were unable to examine specimens from this region, as these were not available through loans from India and no duplicates were available in K or US or other herbaria.

Comments—*Kuruna* differs from *Arundinaria* s. s. by its pachymorph culm bases with short necks, culm leaves usually abaxially hispid, culm leaf girdles ca. 1 mm wide, culm leaf auricles absent and the palea apex biapiculate to acute. The following characters differentiate *Kuruna* from other "*Arundinaria*" groups (*Bergbambos*, African alpine bamboos, *Thamnocalamus* and *Yushania)*: pachymorph culm bases with short

necks, unicaespitose clumps, culm leaf girdles present as a band ca. 1 mm wide, usually abaxially hispid culm leaves, persistent foliage leaf sheaths and complete branch sheathing, palea apex biapiculate (sinus shallow) to acute and undivided, and the absence of both spatheate empty bracts at the base of the synflorescence and subtending bracts (Attigala et al. 2014).

Key to the Species of *Kuruna*

1. Plants clambering or scandent; culm leaf blades reflexed…….. 2

1. Plants erect; culm leaf blades erect to slightly spreading…… 3

2. Supranodal ridge conspicuous; foliage leaf fimbriate auricles present……... *Kuruna debilis*

2. Supranodal ridge inconspicuous; foliage leaf fimbriate auricles absent …… *Kuruna scandens*

3. Primary roots with air canals; nodal line diameter more or less the same as the adjacent internodes; plants of open grasslands (Patana bogs) …….. *Kuruna densifolia*

3. Primary roots lacking air canals; nodal line diameter greater than the adjacent internodes; plants of forest understory or rocky mountain plains ……. 4

4. Culm leaf sheaths abaxially glabrous; foliage leaf blade margins distinctly pale yellow with sharp trichomes on the leading margin …….. *Kuruna serrulata*

4. Culm leaf sheaths abaxially hispid; foliage leaf blade margins green with scabrous leading margin…….. 5

5. Foliage leaf blades more or less cordate, clasping at the base, thick and leathery; glume apex acute..... *Kuruna walkeriana*

5. Foliage leaf blades linear-lanceolate, not clasping at the base, thin and chartaceous; glume apex mucronate…… 6

6. Culm leaf sheath apex more or less horizontal; palea keel wings absent; stigmas 3..... *Kuruna wightiana*

6. Culm leaf sheath apex symmetrically concave; palea keel wings present; stigmas 2……..…..... *Kuruna floribunda*

TABLE1. Morphological comparison of the seven described *Kuruna* species. "?" indicates unknown character states. SI = south India; SL = Sri Lanka.

Character	*K. debilis*	*K. densifolia*	*K. floribunda*	*K. scandens*	*K. serrulata*	*K. walkeriana*	*K. wightiana*
Habitat	Understory	Open grasslands	Understory	Understory	Open rocky plains	Understory	Understory
Distribution	Upper mountain slopes in Central province of Sri Lanka & reported from the Western Ghats region of south India	Horton Plains (wet patanas) of Sri Lanka	Mountains in south central Sri Lanka & Western Ghats region of Kerala, south India	Summit, Mount Piduruthalagala of Sri Lanka	Handapan Ella plains of Sri Lanka	Upper montane zone in Central province of Sri Lanka & Palani Hills, south India	Mountains of south India
Elevation (m)	1,500 –2,500	2,000–2,300	1,000–1,900 (SL) 1,600–2,200 (SI)	2,100–2,500	1,200–1,400	1,400–2,400	1,800–2,500
Vegetative characters							
Primary root air canals	Absent	Present	Absent	Absent	Absent	Absent	Absent
Habit and culm arrangement	Scandent/clambering, culms sparse	Erect, culms dense	Erect & shrubby, culms sparse	Scandent, culms dense	Erect & shrubby, culms dense	Erect & shrubby, culms dense	Erect & shrubby, culms dense
Culm internodes	Hollow	Hollow	Hollow	Hollow	Usually hollow (sometimes solid)	Hollow	Hollow
Culm internode indument	Glabrous	Glabrous	Summit of each internode bearing a ring of purple hairs when young & glabrous with age	Hirsute with white hairs when young & glabrous or scabrid with age	Glabrous	Glabrous	Scaberulous

TABLE1 continued.

Character	*K. debilis*	*K. densifolia*	*K. floribunda*	*K. scandens*	*K. serrulata*	*K. walkeriana*	*K. wightiana*
Internode color when mature	Brownish dark green	Yellowish brown	Light green	Maroon to yellowish maroon	Dark maroon	Brownish dark green	Yellowish brown
Nodal line diameter	Greater than the adjacent internodes	More or less the same as the adjacent internodes	Greater than the adjacent internodes	Greater than the adjacent internodes	Greater than the adjacent internodes	Greater than the adjacent internodes	Greater than the adjacent internodes
Supranodal ridge	Conspicuous	Inconspicuous	Conspicuous	Inconspicuous	Inconspicuous	Inconspicuous	Conspicuous
Culm leaves	Deciduous with development of branches	Persistent	Deciduous	Persistent	Persistent	Deciduous	Deciduous
Culm leaf sheath abaxial surface	Hispid with non-irritating white hairs	Glabrous	Sparsely hirsute with maroon hairs	Hispid with non-irritating dark brown appressed hairs	Glabrous	Hispid with non-irritating white hairs	Hispid with golden brown irritating hairs
Number of leaves per complement	7–10	5–10	6–10	4–8 (10)	6–7	6–14	6–9
Foliage leaf blade shape	Linaer or linear-lanceolate	Narrowly triangular	Lanceolate	Narrowly oblong	Lanceolate	Cordate & clasping at the base	Ovate-lanceolate
Foliage leaf blade margin	Green & glabrous	Green & antrorsely scabrous	Green & sparingly antrorsely scabrous	Green & glabrous (except slightly scabrid toward the base)	Distinctly pale yellow with antrorse sharp trichomes on the leading margin	Green & antrorsely scabrous	Green & scabrous

TABLE1 continued.

Character	*K. debilis*	*K. densifolia*	*K. floribunda*	*K. scandens*	*K. serrulata*	*K. walkeriana*	*K. wightiana*
Foliage leaf margin width (mm)	Ca. 0.1	0.2 (0.2–0.3)	Ca. 0.1	Ca. 0.1	0.3 (0.2–0.5)	0.17 (0.1–0.3)	Ca. 0.1
Foliage leaf length (cm)	5 (3.5–7.4)	3 (2.5–3.8)	13 (6.7–17)	3.1 (2.1–4.5)	9.2 (5.5–12.8)	5.5 (3.2–9.4)	9 (2.5–18.2)
Foliage leaf fimbriate auricles	Present	Absent	Absent	Absent	Absent	Absent	Absent
Foliage leaf sheath summit	Fimbriate	Fimbriate	Fimbriate	Ciliate	Fimbriate with white silky fimbriae	Fimbriate with white silky fimbriae	Fimbriate with light brown long fimbriae
Reproductive characters Synflorescence with pulvinate branches	Present	Absent	Present	Present	?	Present	Present
Number of fertile florets per spikelet	2	1	4–6	2	?	3–4	2–3
Palea apex	Acute	Biapiculate	Biapiculate	Acute	?	Acute	Biapiculate
Palea sulcus	Well developed for the full length	Present only toward the apex	Well developed for the full length	Well developed for the full length	?	Well developed for the full length	Well developed for the full length
Number of stigmas	2	2	2	2	?	2–3	3

KURUNA DEBILIS (Thwaites) Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 200. 2014— *Arundinaria debilis* Thwaites, Enum. Pl. Zeyl. 37. 1864; *Indocalamus debilis* (Thwaites) Alston, Suppl. Fl. Ceylon 6: 342. 1931.—TYPE: SRI LANKA. *C. P. 1* (lectotype designated by Soderstrom and Ellis 1988: PDA; isolectotypes: K!, 3 sheets, US!).

Culms ca. 4.5 m long, ca. 5.5(–4–9) mm in diameter, habit vine-like, scandent or clambering, sparse; internodes 12.9(–7.5–17) cm long, wall thickness 1.4 (1–2) mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.3–0.7, lacuna size 2.7 (1–5) mm, light green when young, becoming brownish dark green with age, glabrous, flattened behind the branch complement on larger culms but the sulcus not prominent; nodal line diameter greater than the adjacent internodes, supranodal ridge conspicuous. Culm leaves deciduous with development of branches; sheaths 12.8(–9–15.2) cm long, abaxially hispid with non-irritating white hairs, apex fimbriate, symmetrically concave; auricles absent; inner ligule a short truncate pubescent rim, papery; blades 3–4 cm long, 3–6 mm wide, narrow, ca. l/5 the length of the sheath, caducous, reflexed. Bud prophyll with white-pilose keels, primary branch producing 2 secondary branches, all 3 developing in an even row and each with several short internodes at the base, initially erect then spreading. Foliage leaves 7–10 per complement; sheaths closely overlapping, summit fimbriate; fimbriate auricles present, more or less equal on both sides of the blade base; inner ligules ca. 0.4 (0.3–0.6) mm long; outer ligule a minute ciliolate rim; blades 5(–3.5–7.4) cm long, 0.4 (0.3–0.6) cm wide, L:W = 11.8(–7.9–17.6), linear or linear-lanceolate, hirtellous on both surfaces, not manifestly tessellate on either surface, apex acuminate, base narrowly cuneate, margins ca. 0.1 mm wide, green, entire, pseudopetiole

ca. 0.5 mm long. Synflorescences 2–6 cm long, 2–3 cm wide, paniculate, with stiff ascending or spreading pulvinate branches. Spikelets 1–1.5 cm long, 2 fertile florets per spikelet, one rudimentary apical sterile floret; glumes early deciduous, ovate, glabrous, apices acute, 8-nerved, glume I 4–5.5 mm long, glume II 5.4–6.8 mm; fertile lemma 7–10.2 mm long, lanceolate-ovate, apex mucronate, 6-nerved, glabrous; palea lanceolate-ovate, 2-keeled, keel wings present, apex acute, sulcus well developed for the full length; lodicules ca. 2 mm long, rhomboid, margins ciliate, the anterior pair many-nerved, nerves extending almost to the tip, the posterior one fewer-nerved and shorter; anthers yellow, ca. 5 mm long, basifixed, developing before the gynoecium; ovary with one style and 2 plumose stigmas. Fruit unknown. Figures 3, 4, 14F.

Distribution and Habitat

The species grows in the understory of the cool upper mountain slopes of the Central Province, Sri Lanka, at elevations of 1,500–2,500 m. Muktesh Kumar (2011) reported that *K. debilis* has been located recently in the Kerala part of the Western Ghats, India, but provided no documentation.

Phenology

This species has been reported to bloom annually (Soderstrom and Ellis 1988), but they themselves regarded this as doubtful. They argued that it is likely that many years occur between the establishment of an individual and its flowering, but that individual clumps in flower may belong to different populations or cohorts. Based on flowering collections from the Horton Plains, the *K. debilis* populations there bloomed in 1968, 1969, 1974, 1975, 1976 and 1978 (no collections in 1977). This species was

collected in flower in 1974 from another location (*Davidse & Sumithraarachchi 8666*), suggesting a possible gregarious flowering event. The 1984 flowering collection is from yet another locality. From the 2010 Attigala collections, only one population from Adam's peak was in bloom (*Attigala et al. 163*) and none of the 2010 Attigala collections of *K. debilis* from Horton Plains were from flowering plants. Therefore, it is difficult to confidently predict the actual flowering interval or behavior of this species.

Comments

Kuruna debilis is the only *Kuruna* species with fimbriate foliage leaf auricles. The deciduous, chartaceous glumes of the spikelets of this species are also distinctive. This is a locally common, scandent bamboo with branches that hang from small trees and other vegetation, sometimes covering them densely.

IUCN Red List category

In 2010, several highly fragmented populations of *K. debilis* were observed. Thus, we suggest that this species should be placed in the *Endangered [EN B1 ab(iii)c(iii)]* category based on the IUCN criteria: Extent of occurrence (EOO) < 5,000 km^2 with severely fragmented locations less than five, continuing decline observed in quality of habitat and extreme fluctuation in the number of subpopulations.

Additional Specimens Examined

SRI LANKA. DISTRICT KANDY: Rangala Hill, E of Kandy, 2,000 m, 03 Nov 1969, *Soderstrom* & *Kulatunge 1771* (PDA, US); trail to Adam's Peak from Moray Estate, 26 Oct 1975, *Sohmer* & *Sumithraarachchi 9896* (MO, PDA, US); Knuckles conservation area, Thangappuwa, 1,400 m, 16 Feb 1995, *Jayasuriya 8694* (PDA);

Northern slopes of Adams peak, along the main trail, 1,650 m, 21 Nov 1974 (fl), *Davidse & Sumithraarachchi 8666* (MO, US); East path up Adams peak, 1,615 m, 26 Oct 1978, *Fosberg 58115* (US, MO); Peak wilderness above Devonford and Maratenne estates, 1,650 m, 15 Aug 1984, *Jayasuruya et al. 2815* (MO); Adams peak (Hatton side), Palabaddala road, N6 48.737, E80 29.869, 1,916 m, 11 Jun 2010, *Attigala et al. 158* (ISC, K, PDA, US); Adams peak (Hatton side), Palabaddala road, N6 48.737, E80 29.869, 1,916 m, 11 Jun 2010, *Attigala et al. 159* (ISC, K, PDA, US); Adams peak (Hatton side), Palabaddala road, N6 48.648, E80 29.776, 1,945 m, 11 Jun 2010, *Attigala et al. 160* (ISC, K, PDA, US); Adams peak (Hatton side), Palabaddala road, N6 48.548, E80 29.893, 2,096 m, 11 Jun 2010, *Attigala et al. 161* (ISC, K, PDA, US); Adams peak (Hatton side), Palabaddala road, N6 48.617, E80 30.032, 2,151 m, 11 Jun 2010 (fl), *Attigala et al. 163* (ISC, K, PDA); Adams peak (Hatton side), Palabaddala road, N6 49.195, E80 29.885, 2,151 m, 11 Jun 2010 (fl), *Attigala et al. 165* (ISC, K, PDA). DISTRICT NUWARA EILYA: Pattipola, on road to Horton Plains, 1,920 m, 08 Jul 1967, *Mueller-Dombois* & *Comanor 67070824* (PDA); Horton Plains at Big World's End Drop, 2,120 m, 17 Oct 1974 (fl), *Davidse 7645* (MO, PDA, US); Pidurutalagala, 2,000 m, 20 Apr 1970, *Gould 13518* (PDA, US); Horton Plains, 2,500 m, 24 Apr 1970, G*ould 13572* (PDA, US); Hakgala, 6 km E of Nuwara Eliya, 2,000 m, 05 May 1970, *Gould* & *Cooray 13748* (PDA, K); Horton Plains, 2,200 m, 30 Oct 1976 (fl), *Jayasuriya 2385* (K, MO, PDA, US), *2388* (MO, PDA, US); 10 km N of Nuwara Eliya, 1,500 m, 17 May 1968 (fl), *Koyama 13622* (PDA); Nuwara Eliya, lower slopes of Pidurutalagala, 1,890 m, 03 Nov 1969, *Soderstrom* & *Kulatunge 1606* (PDA, US); Nuwara Eliya Woods, 1,890 m, 05 Nov 1969 (fl), *Soderstrom* & *Kulatunge 1612* (PDA, US); road from Diyagama Tea Estate to

Horton Plains, *Sohmer* & *Sumithraarachchi 9992* (PDA, MO); Nuwara Eliya, 1,830 m, *s. d., Soderstrom 2550* (US); Agra-Bopat PR. Approach: Bopatalawa, Menik Palama, 1,705 m, 28 Jan 1995, *Jayasuriya 8639* (PDA); Peak Wilderness Sanctuary, trail from Rajamalai to Adam's Peak, 1,725 m, 15 Sep 1995, *Jayasuruya & Karunaratne 9109* (PDA); Kandapola, Sita Eliya forest, Nuwara Eliya Range, 07 Sep 1994, *Jayasuriya 8224* (PDA), Kikiliyamana PR, Nuwara Eliya Range, 09 Oct 1994, *Jayasuriya 8290* (PDA); MAB reserve along road to Horton Plains, 2,400 m, 15 Jul 1978 (fl), *Meijer 1980* (US, MO); Ceylon, *s. d., Thwaites 01* (US); Pidurutalagala mountain, 15 Aug 1978, *Meijer 1959* (US, MO); Northern slopes of Pidurutalagala mountains, 29 Oct 1975, *Sohmer & Sumithraarachchi 10163* (MO, K); Haputala range, Ohiya, 1,840 m, 27 Sep 1978 (fl), *Soderstrom 2553* (K, US); World's end road, Horton Plains, 30 Jul 1975 (fl), *Sumithraarachchi & Sumithraarachchi DBS 953* (K); Horton Plains, 28 Sep 1978 (fl), *Soderstrom 2557* (MO, US); Pidurutalagala mountain, N6 58.824, E80 46.146, 1,958 m, 27 May 2010, *Attigala et al. 120* (ISC, K, PDA); Pidurutalagala mountain, N6 58.716, E80 46.359, 2,001 m, 27 May 2010, *Attigala et al. 121* (ISC, K, PDA); Horton Plains, N6 51.016, E80 48.982, 1,929 m, 28 May 2010, *Attigala et al. 123* (ISC, K, PDA); Horton Plains, N6 50.963, E80 48.881, 1,871 m, 28 May 2010, *Attigala et al. 124* (ISC, K, PDA); Horton Plains, N6 47.643, E80 48.309, 2,130 m, 28 May 2010, *Attigala et al. 130* (ISC, K, PDA); Horton Plains, N6 47.858, E80 49.832, 2,150 m, 28 May 2010, *Attigala et al. 133* (ISC, K, PDA). DISTRICT RATNAPURA: Trail to Gongala, above estate, 1,225 m, 28 Aug 1984 (fl), *Jayasuruya et al. 2899* (MO); Handapan Ella plains, above Eggbirth tea estate, N6 26.736, E80 36.459, 1,251 m, 04 Jun 2010, *Attigala et al. 148*

(ISC, K, PDA, US); Handapan Ella plains, Hellundeniya, near pichchamal aara, N6 26.657, E80 36.102, 1,232 m, 04 Jun 2010, *Attigala et al. 154* (ISC, K, PDA, US**).**

FIG. 3. *Kuruna debilis*: a. Flowering branch (early stage) (×0.6). b. Flowering branch (mature stage) (×0.6). c. Leaf complement (×0.6). d. Leaf ligule (×7). e. Young culm with culm leaves in place (×0.6). f. Culm leaf (lamina abscissed) (×1.2). g. Summit of culm leaf sheath (abaxial view) (×1.2). h. Summit of culm leaf sheath (adaxial view) (×1.2). i. Young culm bud (×3.5). (Illustrations by G. B. Threlkeld, all drawings based on *Soderstrom & Kulatunge 1606*). Note— Supranodal ridge is not distinctly illustrated in I; see Fig. 13F.

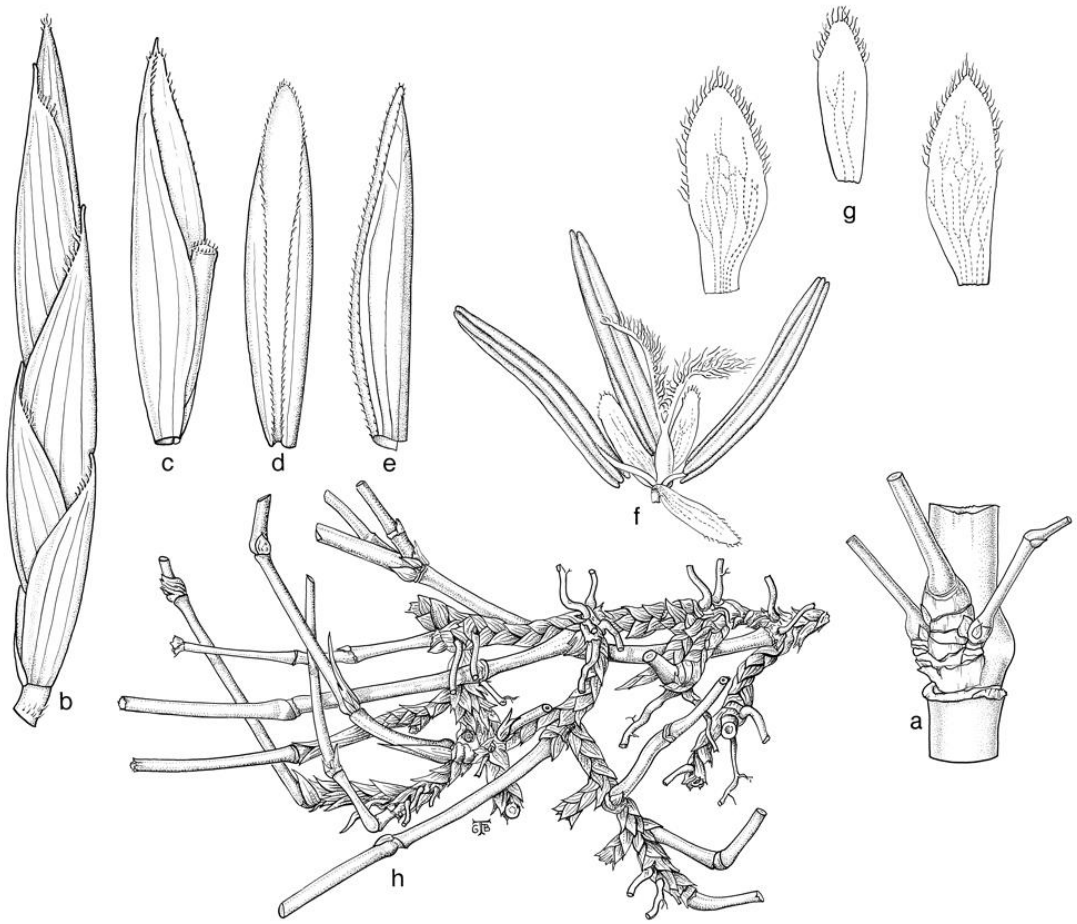


FIG. 4. *Kuruna debilis*: a. Branch complement (×3.5). b. Spikelet (×3.5). c. Lemma with rachilla internode (×7). d. Palea (facing the keels) (×7). e. Palea (lateral view) (×7). f. Flower (×15). h. Rhizome (×0.6). (Illustrations by G. B. Threlkeld, all based on *Soderstrom & Kulatunge 1606*). Note— Supranodal ridge is not distinctly illustrated in a; see Fig. 13F.

KURUNA DENSIFOLIA (Munro) Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 200. 2014— *Arundinaria densifolia* Munro, Trans. Linn. Soc. London 26(1): 32. 1868; *Chimonobumbusa densifolia* (Munro) Nakai, J. Arnold Arbor. 6: 151. 1925.— TYPE: SRI LANKA. "Ceylon" T.T.[Banks] in swamps, *Watson 25* (holotype: K).

Rhizomes with thick primary roots with air cavities, root system completely submerged to ca. 0.33 m deep in standing water. Culms 2–2.5 (3) m tall, 4.6 (3–6) mm in

diameter, erect, dense; internodes 7.2(–3.3–13.3) cm long, wall thickness 1 mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.3–0.7, lacuna size 2.6 (1–4) mm, maroon to maroon-green when young, becoming yellowish brown with age, glabrous; nodal line diameter more or less the same as the adjacent internodes, supranodal ridge inconspicuous. Culm leaves persistent, indurate, appressed to the culm, glabrous; sheaths 4.4 (2.3–6) cm long, abaxially glabrous, apex more or less horizontal; auricles absent; inner ligule a membranous rim less than 0.5 mm long, ciliolate on the margin; blades 1–2 cm long, 2–5 mm wide, narrowly triangular, ca. l/3 to more than 1/2 as long as the sheath, erect to slightly spreading. Bud prophyll brown with white-ciliate keels; primary branch producing 2 secondary branches, all 3 branching in rapid succession giving rise to a cluster of many subequal branches strongly appressed to the culm, erect. Foliage leaves 5–10 per complement; sheaths closely overlapping, summit fimbriate; auricles absent; inner ligules a minute ciliolate membrane; outer ligule a minute ciliolate rim; leaf blades 3 (2.5–3.8) cm long, 0.3 (0.3–0.4) cm wide; L:W = 9.2 (7.8–11), stiff, narrowly triangular, glabrous on both surfaces, tessellate on both surfaces but not always manifest, apex acuminate, base obtuse, margins 0.2 (0.2–0.3) mm wide, green, antrorsely scabrous, trichomes ca. 0.2 mm long, pseudopetiole ca. 1 mm long. Synflorescences ca. 3.5 cm long, ca. 1 cm wide, paniculate, with stiff non-pulvinate branches, glabrous. Spikelets ca. 1 cm long, one fertile floret per spikelet, one rudimentary apical sterile floret; glumes ovate-triangular, apex mucronate, with the single scabrid midnerve, glume I 4.5–6 mm long, glume II 6–7 mm long; fertile lemma 8–10 mm long, lanceolate, apex mucronate, 7-nerved, scabrid; palea 7.5–8.5 mm long, lanceolate-ovate, 2-keeled, keel wings present, apex biapiculate, sulcus present only towards the apex; lodicules ca. 2 mm long,

rhomboid, glabrous except for a few hairs at the summit, the anterior pair with several branching vascular traces, the posterior with fewer traces; anthers pale yellowish, ca. 4–5 mm long, basifixed, developing before the gynoecium; ovary with one style and 2 plumose stigmas. Caryopsis brown, with a persistent style. Figures 5, 6, 14B–C.

Distribution and Habitat

This bamboo is found in the open grasslands of Horton Plains (wet patanas), Sri Lanka, where it often forms dense thickets and grows in cold, standing water at ca. 2,000–2,300 m elevation.

Phenology

Of the specimens examined, flowering specimens were collected in 1890, 1967, 1969, 1970, 1973, 1974 and 1978. Only three collections, from 1975, 1993 and 2010, were without flowers. Almost all of these flowering collections are populations from Horton Plains. Hence, based on these collection records, *K. densifolia* could be an annual bloomer or at least some portion of its populations is always in flower, as has been reported for the high elevation, clump-forming woody bamboo *Chusquea subtessellata* in Costa Rica (Horn and Clark 1992). However, the collection records and label data are insufficient to confidently predict the actual flowering cycle of this species.

Comments

In terms of number of florets per spikelet, this species is the most reduced within the genus, with the spikelet containing a single fertile floret and a reduced floret above (or only the rachilla extension); it is also the only species of the genus that lacks pulvinate synflorescence branches. The roots produce air canals and the dense rhizome bases grow

in cold water. Also, these bamboos of the wet patanas are adapted to the reduction of transpiration and protection of new growth from intense illumination by densely produced foliage, dense branching, densely packed plume-like culms and thick leaves (Soderstrom and Ellis 1988).

Seethalakshmi and Muktesh Kumar (1998) and Muktesh Kumar (2011) reported that *K. densifolia* in India is restricted to south India (Anamudi Hills) and in the southern Western Ghats it grows in Eravikulam and Anamudi from 2,000–2,695 m. However, based on the descriptions and illustrations, we believe that this entity is likely not the same as the *K. densifolia* found in Sri Lanka. Unfortunately, it is difficult to come to a conclusion without seeing the actual Indian *K. densifolia* specimens.

IUCN Red List category

As this species is confirmed to occur only in the open grasslands of Horton Plains, we suggest the *Critically Endangered [CR B1 ab(iii)]* category based on the IUCN criteria: Extent of occurrence (EOO) < 100 km^2 with number of locations 1 and continuing decline observed in quality of habitat.

Additional Specimens Examined

SRI LANKA. DISTRICT NUWARA ELIYA: Horton Plains, Reflection Lake, 2,300 m, 27 Jan 1970 (fl), *Clayton 5486* (PDA, US); along road from Pattipola, 2,175 m, 07 Oct 1967 (fl), *Comanor 451* (PDA, US); Horton Plains, grasslands and forest behind Farr Inn, 2,120 m, 17 Oct 1974 (fl), *Davidse 7600* (MO, PDA, US); meadow N of Farr Inn, 2,300 m, 10 May 1970, *Gould & Cooray 13780* (PDA, US); below resthouse at Ohiya Road, 2,130 m, 09 Jul 1967, *Mueller-Dombois & Comanor 67070915* (US); 18

Mar 1904, *Nock s. n.* (PDA); Horton Plains, 2,195 m, 11 Nov 1969 (fl), *Soderstrom & Kulatunge 1656* (MO, PDA,US); road from Diyagama Tea Estate to Horton Plains-Ohiya Road, 27 Oct 1975, *Sohmer & Sumithraarachchi 9991* (PDA, US), 9993 (MO, PDA, US); Horton Plains, Ohiya road, 28 Oct 1975, *10051* (PDA, MO); Kandepola forest reserve, along loop road, 30 Nov 1973 (fl), *Sohmer et al. 8337* (MO); Horton Plains, 15 Sep 1890 (fl), *Trimen 29* (US); World's End, Bogawanthalawa foot path, 07 Oct 1973, *Waas 169* (PDA, US); Horton Plains, 2,200 m, 29 Mar 1993, *Weerasinghe & Jayasekara s.n.*(PDA); Horton Plains, 2,160 m, 27 Sep 1978 (fl), *Soderstrom 2556* (US); MAB Reserve along road to Horton Plains, 2,300 m, 15 Jul 1978 (fl), *Meijer 1986* (MO, US); Horton Plains, 2,000 m, 04 Dec 1970, *Larsen AAU70-29474* (MO); Horton Plains, N6 50.341, E80 48.729, 2,174 m, 29 May 2010, *Attigala et al. 125* (ISC, K, PDA); Horton Plains, N6 50.300, E80 48.660, 2,173 m, 29 May 2010, *Attigala et al. 126* (ISC, K, PDA); Horton Plains, N6 49.532, E80 48.372, 2,142 m, 29 May 2010, *Attigala et al. 127* (ISC, K, PDA); Horton Plains, N6 47.986, E80 48.386, 2,120 m, 29 May 2010, *Attigala et al. 128* (ISC, K, PDA); Horton Plains, N6 47.913, E80 48.334, 2,119 m, 29 May 2010, *Attigala et al. 129* (ISC, K, PDA); Horton Plains, N6 47.643, E80 48.309, 2,120 m, 29 May 2010, *Attigala et al. 130* (ISC, K, PDA); Horton Plains, N6 48.440, E80 48.391, 2,135 m, 29 May 2010, *Attigala et al. 131* (ISC, K, PDA); Horton Plains, N6 47.666, E80 49.556, 2,119 m, 29 May 2010, *Attigala et al. 132* (ISC, K, PDA).

FIG. 5. *Kuruna densifolia*: a. Leafy branch (×0.6). b. Whorl of branches on young culm (×0.6). c. Branch whorl detail (×0.6). d. Leaf complement (×0.6). e. Leaf ligule (×7). f. Culm leaf (adaxial view to show ligule) (×1.7). g. Culm leaf (abaxial view) (×1.7). h. Mid-culm bud (×1.7). i. Flowering branches (×1.1). (Illustrations by G. B. Threlkeld, based on *Soderstrom & Kulatunge 1656*).

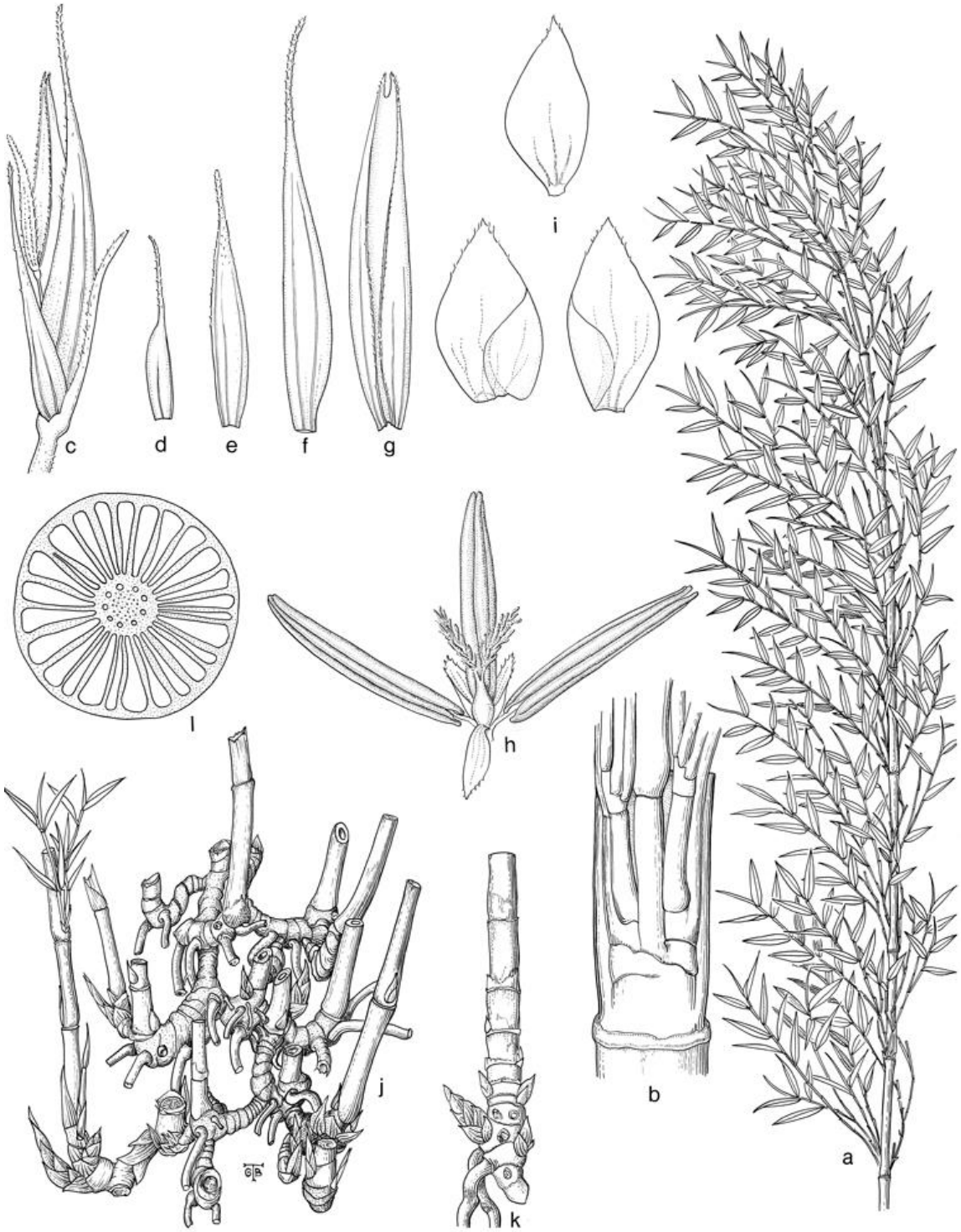


FIG. 6. *Kuruna densifolia*: a. Vegetative culm (×0.3). b. Branch complement (×3.5). c. Spikelet (×7). d. Lower glume (×7). e. Upper glume (×7). f. Lemma (×7). g. Palea (×7). h. Flower (×7). i. Lodicules (×l.5). j Rhizome (×0.6). k. Rhizome bud pattern (×0.6). i. Primary root (cross section) (×3.5). (Illustrations by G. B. Threlkeld, based on *Soderstrom & Kulatunge 1656*).

KURUNA FLORIBUNDA (Thwaites) Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 200. 2014— *Arundinaria floribunda* Thwaites, Enum. Pl. Zeyl. 375. 1864; *Indocalamus floribundus* (Thwaites) Nakai, J. Arnold Arbor. 6: 148. 1925.— TYPE: SRI LANKA. Maturatte district, 1520 m, *Thwaites s. n.*, in 1853, *C. P. 2624* (holotype: PDA; isotypes: K! 2 sheets, US!).

Culms ca. 2.5 m tall, 6.2(–3–10) mm in diameter, erect, shrubby, sparse; internodes 18.4(–7–30) cm long, wall thickness 1 mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.2–0.7, lacuna size 4.2(–1–8) mm, light green and spotted with purplish black specks when young, becoming lighter in color with age, summit bearing a ring of purple hairs when young, glabrous when older; nodal line diameter greater than the adjacent internodes, supranodal ridge conspicuous. Culm leaves deciduous; sheaths 10.5(–7.1–16.1) cm, abaxially sparsely hirsute throughout with maroon hairs, apex symmetrically concave; auricles absent; inner ligule a short membrane, ca. 0.5 mm long, abaxially puberulent; blades 3 cm long, 2–3 mm wide, ca. linear-lanceolate, ca. l/3 –1/4 as long as the sheath, initially erect, becoming reflexed. Primary branch producing 2 secondary branches, followed by other branches from these axes to form a whorl of usually 5 more or less equal branches, the latter occasionally rebranching but at maturity the whole branch complement not crowded. Foliage leaves 6–10 per complement; sheath summit fimbriate; auricles absent; inner ligule a minute short rim; outer ligule a minute ciliolate rim; blades 13(–6.7–17) cm long, 1.3 (0.9–1.8) cm wide; L:W = 10.9(–6.7–14.2), lanceolate, glabrous on both surfaces, tessellate on both surfaces, apex acuminate, base attenuate, margins ca. 0.1 mm wide, green, glabrous or

sparingly antrorsely scabrous, trichomes ca. 0.1 mm long, pseudopetiole 2.6 (1–4) mm long. Synflorescences 15–20 cm long, 8–10 cm wide, paniculate, branches pulvinate, glabrous, spreading. Spikelets 2.5–3.5 cm long, 4–6 fertile florets per spikelet, one rudimentary apical sterile floret; glumes glabrous, with a few cilia on the upper edges, apex mucronate, glume I 3.8–5.6 mm long, ovate-triangular, 5-nerved, glume II 5.5–6.1 mm long, ovate-lanceolate, 7-nerved; fertile lemma 8.5–10 mm long, ovate-lanceolate, apex mucronate, 7 or 8-nerved, scabrous; palea 3.8–6.3 mm long, lanceolate, 2-keeled, keel wings present, apex biapiculate, sulcate between the keels, sulcus well developed for the full length; lodicules 1.5–2 mm long, anterior pair rhomboid, narrowed at the base, posterior lanceolate, all apically ciliate; anthers orange-yellow, ca. 4.3–6 mm long, basifixed; ovary not found. Fruit unknown. Figures 7, 8, 14A.

Distribution and Habitat

This bamboo is distributed in the understory of montane forests of south-central Sri Lanka at ca. 1,000–1,900 m elevation. Individual plants of *K. floribunda* were scattered throughout the closed forest associated with *Cupressus.* This species is also found in the Western Ghats region of Kerala (Rao 1914) and Munnar on the way to Anamudi and Berijam from 1,600–2,200 m of south India (Muktesh Kumar 2011).

Phenology

The only flowering specimen collected was from 1910 and other flowering specimens have not been seen. Thus, at least in the most recent two decades this species has remained vegetative and we hypothesize that it has an extended flowering cycle.

Comments

The presence of a thick, prominent supranodal ridge and small clumps with culms 2–3.5 m tall with the internodes somewhat scabrous and speckled with purplish markings are the most diagnostic characteristics of this species. As noted in Soderstrom and Ellis (1988) and based on our own collections, this species does not occur in abundance. We were able to collect only two populations in 2010, which were persisting in deforested regions.

IUCN Red List category

Based on our observations in 2010, we suggest the *Critically Endangered [CR B1 ab(i,iii)]* category for *K. floribunda* based on the IUCN criteria: Extent of occurrence (EOO) < 100 km^2 with number of locations 1, continuing decline observed in EOO and quality of habitat.

Additional Specimens Examined

SRI LANKA. DISTRICT BADULLA: between Ohiya and Boralanda, 1,890 m, 13 Nov 1969, *Soderstrom & Kulatunge 1658* (K, PDA, US). DISTRICT RATNAPURA: Sinharaja, Handapan ella plains, above Ilumbakanda Estate, 1,250 m, 14 Jan 1993, *Jayasuriya & Wijesinghe 7094* (PDA); Wewiyathalawa-Kithulgala, N7 03.049, E80 24.009, 1,167 m, 20 Jun 2010, *Attigala et al. 135* (ISC, K, PDA); Wewiyathalawa-Kithulgala, N7 02.967, E80 24.085, 1,212 m, 20 Jun 2010, *Attigala et al. 139* (ISC, K, PDA); Handapan Ella OSF, approach from Botivatenna on Rakwana- Pothupitiya Road, 1,100 m, 13 Feb 1994, *Jayasuruya 8024* (PDA). DISTRICT KEGALLE: Amanawala-Ampane PR, Kitulgala Range, 1,100 m, 22 Nov 1994, *Jayasuriya & Wijesinghe 8574*

(PDA). DISTRICT NUWARA ELIYA: Haputala range, forests near Ohiya, 1,780 m, 27 Aug 1978, *Soderstrom 2555* (US)**.** DISTRICT NOT GIVEN: top of Naminabuli, 24 Oct 1910 (fl), *Willis 103* (US).

FIG. 7. *Kuruna floribunda*: a. Leaf complement (×0.6). b. Leaf ligule (×3.5). c. Culm leaf in place (×0.6). d. Culm leaf sheath (outside view) (×3.5). e. Bud on young culm (×1.7). f. Branching, early stage (×1.7). g. Branch complement (×1.7). (Illustrations by G. B. Threlkeld, based on *Soderstrom & Kulatunge 1658*). Note— No distinct constriction at the apex of the foliage leaf blade, as shown in a, occurs in the actual species, and the supranodal ridge is not distinctly illustrated in e; see Fig. 13A for supranodal ridge.

FIG. 8. *Kuruna floribunda*: a. Inflorescence (×0.6). b. Spikelet (glumes missing) (×3.5). c. Lower glume (×7). d. Upper glume (×7). e. Lemma (×7). f. Palea (×7). g. Flower (×7). h. Lodicules (×15). i. Rhizome (×0.6). (Illustrations by G. B. Threlkeld, all based on *Jowitt s.n.*, 28 Feb 1902, except i, based on *Soderstrom & Kulatunge 1658*).

KURUNA SCANDENS (Soderstrom & Ellis) Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 200. 2014— *Arundinaria scandens* Soderstrom & Ellis, Smithsonian Contr. Bot. 72: 20. 1988.— TYPE: SRI LANKA. summit of Pidurutalagala, Sep 1881, *Beddome s. n.* (Holotype: PDA, US 2903434 (frag.)!).

Culms ca. 7–8 m long, ca. 3.6(–2.5–7) mm in diameter, at first erect, then arching and clambering, dense, clumps of 100–200 culms; internodes 9.8 (8.7–10.8) cm long, wall thickness 1.1 (1–2) mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.5–0.8, lacuna size 1.4(–0.5–3) mm, light green when young, becoming maroon to yellowish maroon with age, hirsute with white hairs when young, glabrous or scabrid with age; nodal line diameter greater than the adjacent internodes, supranodal ridge inconspicuous. Culm leaves persistent, sheaths 5.3 (3.8–6.5) cm long, abaxially hispid with non-irritating dark brown appressed hairs, apex symmetrically concave; auricles absent; inner ligule a rounded membrane ca. 1.7 mm long with an irregularly toothed margin; blades 10–12 cm long, ca. 2.5 mm wide, narrowly oblong, ca. l/8–1/10 the length of the sheath, caducous, reflexed. Bud prophyll with dark brown pilose keels; primary branch producing 3 or 4 secondary branches developing shortly after the main axis has elongated, the secondary branches in turn producing tertiary branches at their nodes, the whole ultimately resulting in a dense cluster of long, widely divergent branches, enveloped at the base by the persistent sheath. Foliage leaves 4–8 (10) per complement; sheaths closely overlapping, summit ciliate; auricles absent; inner ligule 0.9 (0.7–1.2) mm long, an irregular, erose membrane, glabrous; outer ligule absent; blades 3.1 (2.1–4.5) cm long, 1.0 (0.5–1.5) cm wide; L:W = 3.7 (2.5–5.3), narrowly oblong, glabrous, strongly tessellate on both surfaces, apex acute, base rounded, margins ca. 0.1 mm wide,

green, entire (except slightly scabrid toward the base), pseudopetiole 1.2 (1–2) mm long. Synflorescences 6–7 cm long, ca. 6 cm wide, paniculate, with stiff ascending or spreading pulvinate branches, glabrous. Spikelets ca. 1.2 cm long, 2 fertile florets per spikelet, one rudimentary apical sterile floret; glumes ovate-triangular, abaxially glabrous, adaxially puberulent on the upper part, apices mucronate, glume I 2.7–3.6 mm long, glume II 3.9–4.5 mm; fertile lemma 6.3–7.5 mm long, ovate-lanceolate, apex mucronate, 7-nerved, abaxially glabrous, adaxially puberulent on the upper part; palea broad, strongly 2-keeled, keel wings present, 2 nerves in each wing, 3 nerves between the keels, apex acute, sulcus well developed for the full length; lodicules 1.6–1.7 mm long, margins ciliate; anthers pale yellow, ca. 5 mm long; ovary with one style and 2 plumose stigmas. Fruit unknown. Figures 9, 14E.

Distribution and Habitat

This species is endemic to the summit of Mount Piduruthalagala, Sri Lanka, where it occurs in the forest understory from ca. 2,100–2,500 m (summit).

Phenology

Flowering specimens were collected in 1887 and a collection by Thwaites (*Thwaites 3860*) is of unknown date. However, records show a collection of *Panicum stenostachyum* Thwaites (*Thwaites 3845*) from Sri Lanka in 1964 (Tropicos®: http://www.tropicos.org), so we infer that the *Thwaites 3860* specimen could have been collected around 1964. Though there are limited collections of this species, none of the most recent collections (from 1978 and 2010) consist of flowering material and thus, it seems likely that this species also flowers at long intervals.

Comments

Soderstrom and Ellis (1988) found that this species replaces *K. debilis* on Mount Pidurutalagala at elevations above 2,100 m. But according to our observations, *K. scandens* is now restricted only to the peak of Mount Pidurutalagala, due to an excessive amount of human interference with its habitat, and this single population seems to be declining rapidly.

IUCN Red List category

Since this species is endemic to Mount Piduruthalagala, Sri Lanka, we propose the *Critically Endangered [CR B1 ab(i,iii,v)]* category for *K. scandens* based on the IUCN criteria: Extent of occurrence (EOO) < 100 km^2 with number of locations 1, continuing decline observed in EOO, quality of habitat and number of mature individuals.

Additional Specimens Examined

SRI LANKA. DISTRICT KANDY: Knuckles Mountains, Mar 1887 (fl), *Ferguson 312* (US). DISTRICT NUWARA ELIYA: Piduruthalagala, 2,347 m, 26 Oct 1978, *Soderstrom & Kulatunge 1608* (K, PDA, US); Piduruthalagala, 2,650 m, 16 Feb 1978, *Clayton 5756* (PDA, US); Piduruthalagala, 2,347 m, 26 Oct 1978, *Soderstrom 2550* (K, US); Ceylon, *s. d.* (fl), *Thwaites 3860* (US); Piduruthalagala mountain top, N7 02.967, E80 24.085, 2,511 m, 31 Jun 2010, *Attigala et al. 166* (ISC, K, PDA); Piduruthalagala mountain top, N7 02.967, E80 24.085, 2,511 m, 31 Jun 2010, *Attigala et al. 167* (ISC, K, PDA).

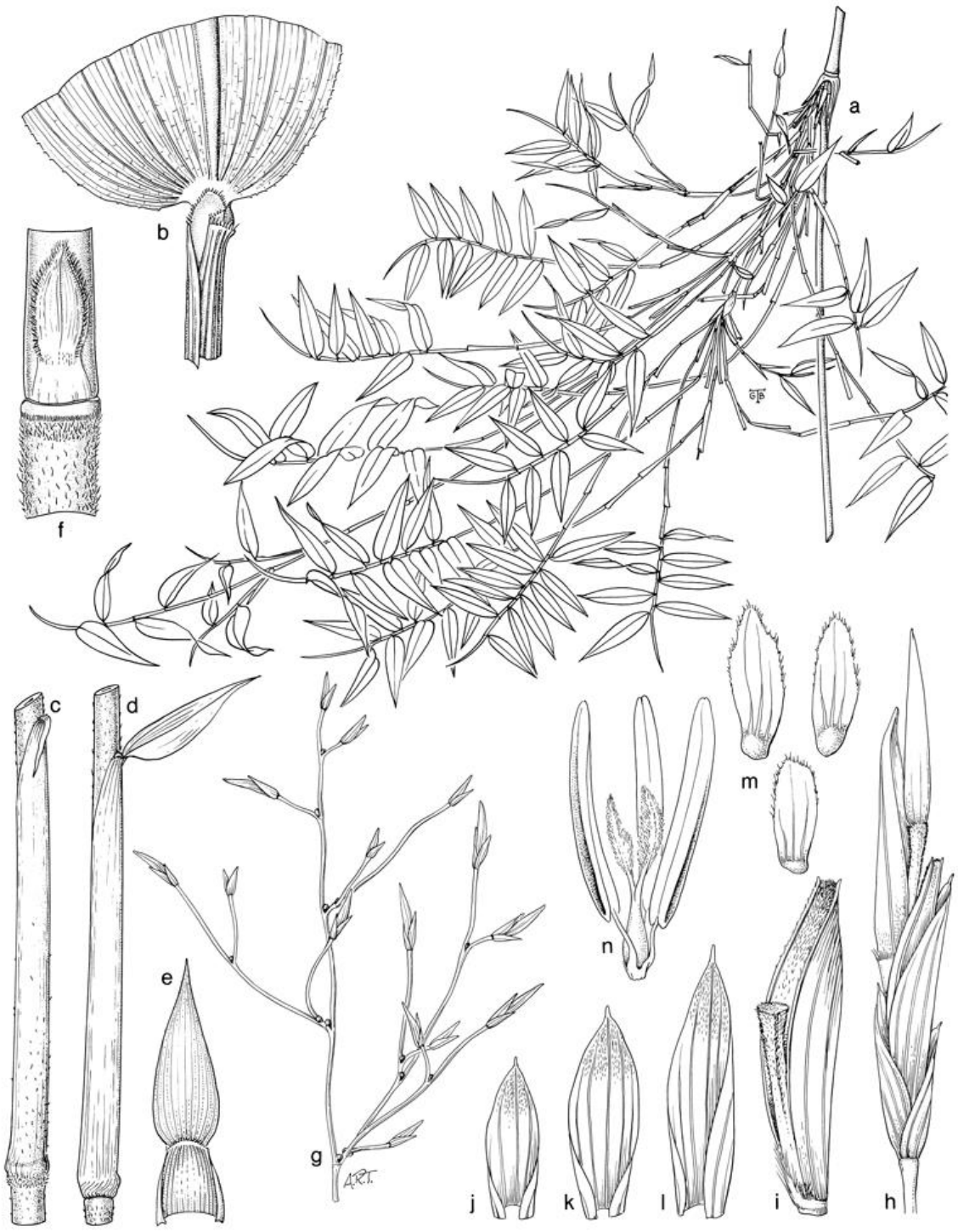


FIG. 9. *Kuruna scandens*: a. Leafy branch habit (×0.3). b. Foliage leaf ligule (×3.4). c. Young culm leaf in place (×0.6). d. Older culm leaf in place (×0.6). e. Culm leaf, adaxial view to show ligule (×0.6). f. Young culm bud (×1.1). g. Inflorescence (×1.1). h. Spikelet (×7). i. Floret, showing palea and rachilla segment (×8.5). j. Lower glume (×8.5). k. Upper glume (×8.5). l. Lemma (×8.5). m. Lodicules (×14). n. Androecium and gynoecium (×8.5). (Illustrations by G. B. Threlkeld, a-f based on *Soderstrom & Kulatunge 1608*, g-n on *Beddome s.n.*, Sep 1881). Note— Palea tips are shown as biapiculate in i, but they are actually acute.

Kuruna serrulata Attigala, Kathriarachchi & L. G. Clark, sp. nov. — TYPE: SRI LANKA. Handapan Ella plains, above the Eggbirth estate, N6 26.739, E80 36.470, 1,245 m, 04 Jan 2010, *L. Attigala, N. de Silva and A. de Silva 149* (Holotype: PDA!, isotypes: ISC!, K!)

Culms 1.0–1.5 m long, 7 (5–10) mm in diameter, erect, shrubby, dense; internodes 12.9(–9.2–24) cm long, wall thickness 1 (1–3) mm, terete, internodes usually hollow (sometimes solid), ratio of 2 times wall thickness: culm diameter 0.2–1, lacuna size 5 (2–6) mm, greenish brown and spotted with black specks when young, becoming dark maroon with age glabrous; nodal line diameter greater than the adjacent internodes, supranodal ridge inconspicuous. Culm leaves persistent; sheaths 5.8 (5–7.3) cm long, abaxially glabrous, apex more or less horizontal; auricles absent; inner ligule a minute ciliate membrane; blades 1.8–3.4 cm long, ca. 0.5 mm wide, narrowly ovate, ca. l/2 the length of the sheath, erect to slightly spreading. Primary branch producing 2 lateral branches, followed by rebranching of the lateral branches giving rise to a complement of 10–20 subequal branches. Foliage leaves 6–7 per complement; sheath summit fimbriate with white silky fimbriae; auricles absent; inner ligules minute, truncate; outer ligule a short inconspicuous rim; blades 9.2(–5.5–12.8) cm long, 1 (0.6–1.4) cm wide; L:W = 7.2(–5.8–10.8), lanceolate, thick and leathery, strongly tessellate on both surfaces, apex acuminate, base obtuse, margins 0.3 (0.2–0.5) mm wide, distinctly pale yellow with antrorse sharp trichomes on the leading margin, trichomes ca. 0.6 mm long, pseudopetiole 1.6 (1–3) mm long. Synflorescences unknown. Figure 10.

Etymology

Kuruna serrulata is named for the presence of distinct sharp trichomes on the leading margin of the foliage leaf blades.

Distribution and Habitat

The species is distributed mainly in open rocky montane plains with shallow soil cover of Handapan Ella plains of the southern province of Sri Lanka, at an elevation of ca. 1,200–1,400 m.

Phenology

All collections of this species to date are vegetative.

Comments

This species is unique due to the presence of distinct sharp trichomes on the leading margin of the foliage leaves and distinctly pale yellow foliage leaf margins. Further, though this species usually possesses hollow internodes, it sometimes has solid internodes too. However, we were unable to identify a clear pattern between hollow and solid culms. Although this species resembles both *K. walkeriana* and *K. floribunda* in certain respects, we here recognize it as a new species because of its distinct habitat (open rocky montane plains) and a unique combination of characters, particularly the unusual color and serration of the foliage leaf blade margins.

IUCN Red List category

We saw several fragmented populations of *K. serrulata* in 2010. Hence, we suggest the *Endangered [EN B1 ab(i, iii)]* category based on the IUCN criteria: Extent of

occurrence (EOO) < 5000 km^2 with severely fragmented locations less than five and continuing decline observed in quality of habitat.

Additional Specimens Examined

Hayes Estate- Gongala. On top of the rocky mountain, N6 23.099, E80 39.344, 1,307 m, 03 Jan 2010, *Attigala et al. 146* (ISC, K, PDA); Handapan Ella plains, above the Eggbirth estate, N6 26.721, E80 36.236, 1,225 m, 04 Jan 2010, *Attigala et al. 150* (ISC, K, PDA); Handapan Ella plains, Hellundeniya, near pichchamal ara, N6 26.657, E80 36.102, 1,232 m, 04 Jan 2010, *Attigala et al. 152* (ISC, K, PDA); Handapan Ella plains, Hellundeniya, near pichchamal ara, N6 26.657, E80 36.102, 1,232 m, 04 Jan 2010, *Attigala et al. 153* (ISC, K, PDA).

KURUNA WALKERIANA (Munro) Attigala, Kathriarachchi & L. G. Clark, Phytotaxa 174 (1): 200. 2014— *Arundinaria walkeriana* Munro, Trans. Linn. Soc. London 26(1): 21. 1868—TYPE: *Mrs. Walker 96* (lectotype designated by Soderstrom and Ellis 1988: K, isolectotype: K!)

Culms ca. 2 m long, 4.1 (3–6) mm in diameter, erect, shrubby, dense; internodes 10.2(–5.5–15.3) cm long, wall thickness 1.2 (1–1.5) mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.5–0.8, lacuna size 1.8 (1–3) mm, light green when young, becoming brownish dark green with age, glabrous; nodal line diameter greater than the adjacent internodes, supranodal ridge inconspicuous. Culm leaves deciduous; sheaths 5.9 (5.4–6.5) cm, abaxially hispid with non-irritating white hairs, at the base densely hirsute and remaining as a hairy ring after the sheath falls, apex symmetrically concave; auricles absent; inner ligule membranous; blades 2–3 cm long, ca. 0.5 mm wide, narrowly ovate, ca. l/3 the length of the sheath, reflexed. Primary branch producing 2

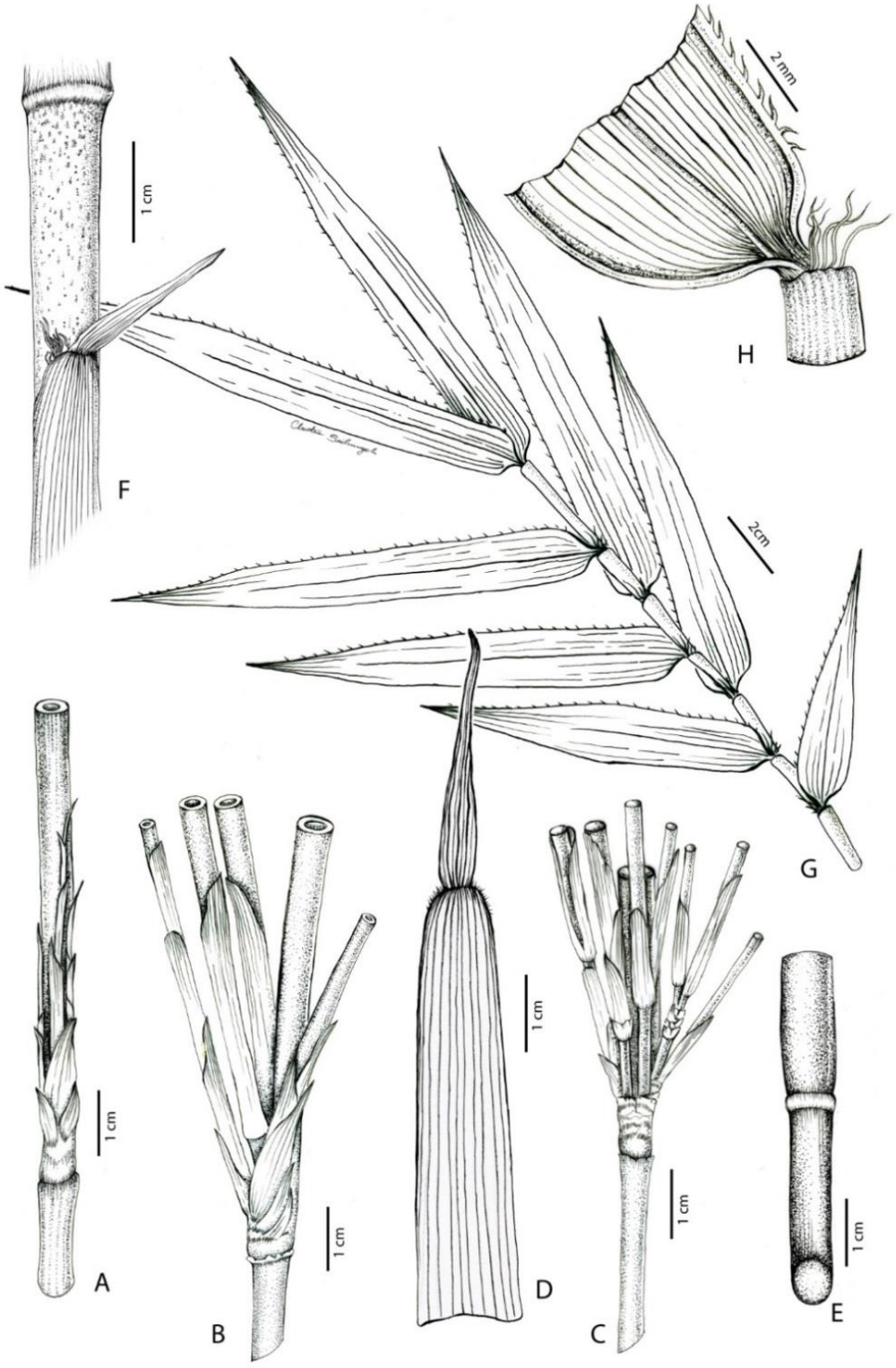


FIG. 10. *Kuruna serrulata*: A. Branching, early stage. B. Intermediate branch complement. C. Mature branch complement. D. Culm leaf. E. Solid culm. F. Older culm leaf in place. G. Leaf complement. H. Foliage leaf summit and leaf margin trichomes. (Illustrations by C. Sidorowych, A based on *Attigala et al. 150*, C & F based on *Attigala et al. 149*, B, D–E, G-H based on *Attigala et al. 152*).

lateral branches, quickly followed by the development of tertiary branches to produce a complement of up to 30 branches, these rarely re-branching, but continuing in length to ca. 40 cm, with 10–20 leaves, with production of new leaves and shedding of old blades, covered by only hard, persistent, overlapping sheaths. Foliage leaves 6–14 per complement; sheath summit fimbriate with white silky fimbriae; auricles absent; inner ligule 1.1 (0.7–1.2) mm long; outer ligule absent; blades 5.5(–3.2–9.4) cm long, 1.5 (0.8–2.4) cm wide; L:W = 3.9 (2.4–5.8), more or less cordate, glabrous, clasping at the base, thick and leathery, tessellate on both surfaces, apex acute, base rounded, margins 0.2 (0.1–0.3) mm wide, green, antrorsely scabrous, trichomes ca. 0.1 mm long, pseudopetiole 1.5(1–3) mm long. Synflorescences 20–30 cm long, 20–30 cm wide, paniculate, smaller on the leafy branches, the branches glabrous, pulvinate, spreading. Spikelets 14–20 mm long, 3–4 fertile florets per spikelet, one rudimentary apical sterile floret; glumes ovate-lanceolate, abaxially glabrous, ciliate on the margins, apices acute, 7-nerved, glume I ca. 5.8 mm long, glume II ca. 6.5 mm; fertile lemma ca. 7.3 mm long, ovate, apex acute, 9-nerved, ciliate on the upper margin; palea lanceolate-ovate, 6–7 mm long, 2-keeled, 2 broad keel wings present, apex acute, sulcus well developed for the full length; lodicules ca. 1.4 mm long, lanceolate, upper margins ciliate, many-nerved; anthers orange, ca. 5 mm long; ovary with a one style and 2–3 plumose stigmas. Fruit an ellipsoid caryopsis, ca. 5 mm long with a short beak (the persistent base of the style), embryo basal, ca. l/5 the length of the fruit. Figures 11, 12, 14D.

Distribution and Habitat

The species is endemic to the upper montane zone of the Central Province of Sri Lanka and occurs as an understory species at ca. 1,400–2,400 m elevation. It is also

reported from southern India, particularly in the Palani Hills, Agasthyamala and Eravikulam (Seethalaksmi and Muktesh Kumar 1998; Muktesh Kumar 2011).

Phenology

Flowering specimens have been collected only in September of 1980 and none of the other collections (from 1969, 1970, 1977, 1985 and 2010) was flowering. Thus, this species likely flowers at long intervals.

Comments

The thick, leathery cordate-shaped clasping foliage leaves are characteristic of this species. *Kuruna walkeriana* was misidentified as *K. wightiana* in several herbarium specimens from Sri Lanka. To our knowledge and from previous studies (Soderstrom and Ellis 1988; Muktesh Kumar 2011), *K. wightiana* is only reported from southern India (see below).

IUCN Red List category

During 2010, we found *K. walkeriana* only in the Adam's peak region. Thus, we suggest the *Critically Endangered [CR B1 ab(i,iii)]* category for this species based on the IUCN criteria: Extent of occurrence (EOO) < 100 km^2 with number of locations 1 and continuing decline observed in EOO and quality of habitat.

Additional Specimens Examined

SRI LANKA. DISTRICT KANDY: Knuckles Mountains, 1,800 m, 19 May 1970, *Gould & Balakrishnan 13897* (PDA, US); Knuckles, on mountain range N. of Kaluphane, 1,740 m, 17 Sep 1977, *Meijer 1342* (US). DISTRICT NUWARA ELIYA: on the way to Adam's Peak from Halton side, near Edikatupane, 2,330 m, 05 Dec 1969,

Soderstrom & Kulatunge 1772 (K, PDA, US); Above Indikatupahana, southern slope of Peak Wilderness Sanctuary above Sri Palabaddala, 1,450 m, 01 Mar 1985, *Jayasuriya & Gunatilleke 3175* (PDA, MO); Ceylon, 2,195 m, 15 Sep 1890 (fl), *Gamble* (K); Adam's peak, road to Palabaddala, near indikatupahana, 2,151 m, 12 Jun 2010, *Attigala et al. 162* (ISC, K, PDA); Adam's peak, road to Palabaddala, near indikatupahana, 2,091 m, 12 Jun 2010, *Attigala et al. 162B* (ISC, K, PDA).

Kuruna wightiana (Nees) Attigala, Kathriarachchi & L. G. Clark, comb. nov.

Arundinaria wightiana Nees, Linnaea 9(4): 482. 1834. *Indocalamus wightianus* (Nees) Nakai, J. Arnold Arbor. 6: 149. 1925. *Yushania wightiana* (Nees) R.B. Majumdar, Fl. Ind. Enum.: Monocot. 283. 1989. — TYPE: INDIA. Nilgiri, *Wright 1797* (lectotype designated by Muktesh Kumar 2011: CAL, isolectotype: K!, 2 sheets)

Culms ca. 8 m long, ca. 9(–5–11) mm in diameter, erect, shrubby, dense; internodes ca. 30 cm long, wall thickness 2 (1–2) mm, terete, hollow, ratio of 2 times wall thickness: culm diameter 0.4, lacuna size 5(–3–7) mm, dark green when young, becoming yellowish brown with age, scaberulous; nodal line diameter greater than the adjacent internodes, supranodal ridge conspicuous. Culm leaves deciduous; sheaths 13.2(–8.8–19.2) cm long, abaxially hispid with golden brown irritating hairs, at the base densely hirsute, apex more or less horizontal; blades 3.8 (2.5–4) cm long, 0.5 (0.3–0.6) cm wide, narrowly ovate, ca. l/5–1/4 the length of the sheath, reflexed; auricles absent; inner ligule short ciliolate. Primary branch producing 2 (3) lateral branches, quickly followed by development of basal buds to produce ca. 6-12 smaller subequal branches [sometimes up to 50 subequal branches fide Muktesh Kumar (2011)]. Foliage leaves 6–9 per complement; sheath

FIG. 11. *Kuruna walkeriana*: a. Culm leaf in place (×1.2). b. Leaf complement (×0.6). c. Culm leaf (adaxial view) (×1.7). d. Rhizome bud pattern (×1.2). e. Mature branch complement (×3). f. Leaf ligule, side view (×7). g. Bud on new culm (×3). h. Leaf ligule, front view (×6). i. Young branch complement (×1.2). (Illustrations by G. B. Threlkeld, based on *Soderstrom & Kulatunge 1772*).

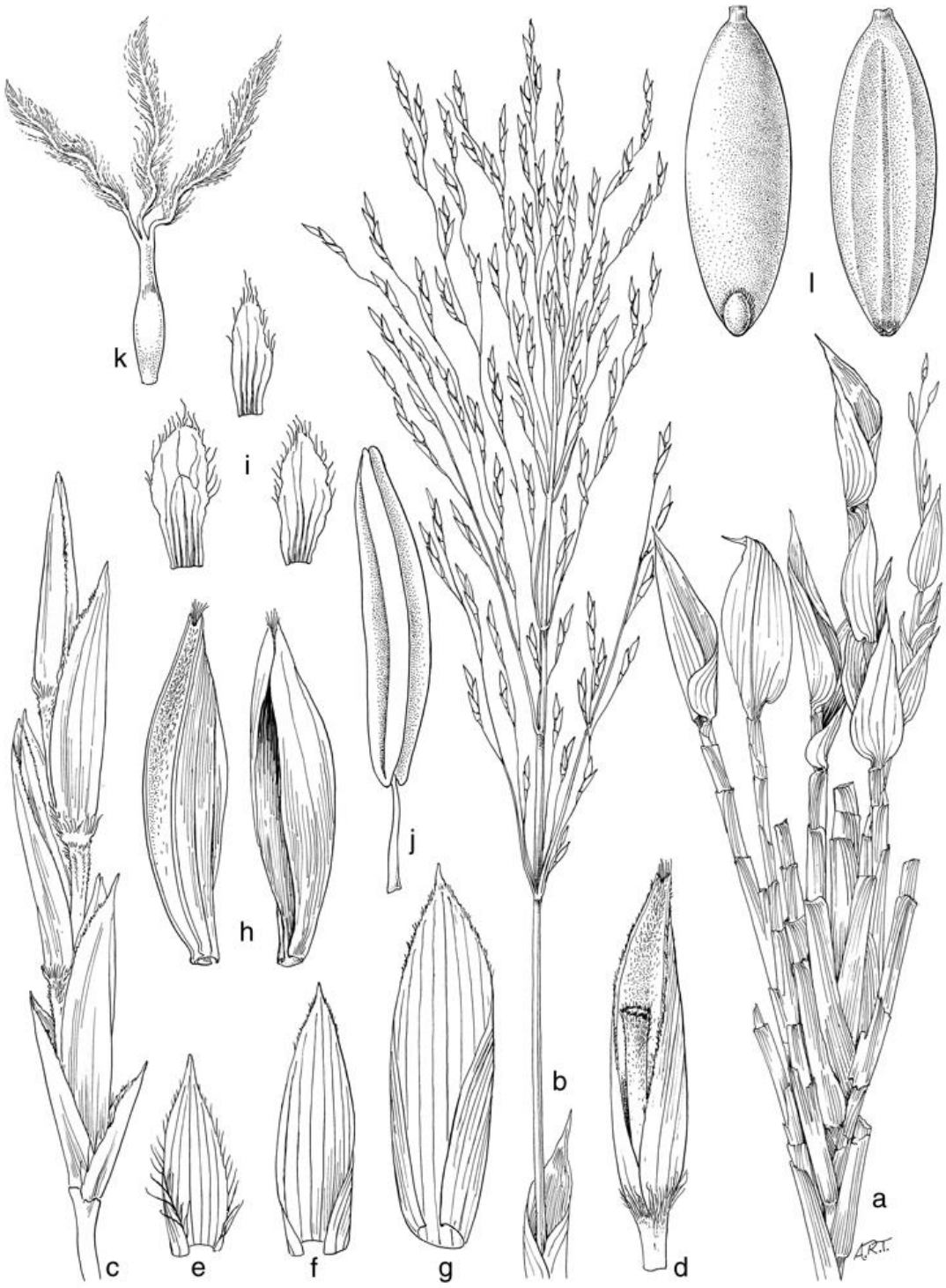


FIG. 12. *Kuruna walkeriana*: a. Vegetative branches (×0.6). b. New inflorescence recently emerged from subtending sheath (×0.6). c. Spikelet (×7). d. Floret (×11). e. Glume I (×11). f. Glume II (×11). g. Lemma (×11). h. Palea, side view showing keels and frontal view (×11). i. Lodicules, lower anterior pair and upper posterior (×1.5). j. Anther (×15). k. Gynoecium (×15). l. Caryopsis, embryo view and hilum view (×11). (Illustrations by G. B. Threlkeld, a, b, and l based on *Ferguson s.n.* in 1887, Knuckles Mountains; all others based on specimens from Dumbanagala, Rangala, 28 Sep 1888, *s. coll.*). Note— Palea tips are shown as biapiculate in h, but they are actually acute.

summit fimbriate with light brown long (ca. 1 cm) fimbriae; auricles absent, inner ligules truncate; outer ligule a minute glabrous rim; blades 9(–2.5–18.2) cm long, 1.2 (0.6–1.9) cm wide; L:W = 7.6(–4.2–9.6), ovate-lanceolate, tessellate on both surfaces, apex acuminate, base obtuse, margins ca. 0.1 mm wide, green, antrorsely scabrous, trichomes ca. 0.2 mm long, pseudopetiole 1.5(–0.5–3) mm long. Synflorescence 10–15 cm long, –3–12 cm wide, paniculate, branches with prominent pulvini, spreading. Spikelets 1.5–2 cm long, 2–3 fertile florets per spikelet, one rudimentary apical sterile floret; glumes oblong, abaxially glabrous, ciliate at the tips, apices acute, 6–7-nerved, glume I ca. 3 mm long, glume II ca. 3.5 mm; fertile lemma 4–5 mm long, ovate, apex mucronate, 7–11 nerved; palea lanceolate, 0.4–0.5 cm long, 2-keeled, keel wings absent, ciliate on the keels, apex biapiculate, sulcus well developed for the full length; lodicules ca. 1.4 mm long, upper margins ciliate, 3–7 nerved; stamens 3, brown, basifixed; ovary with a one style and 3 plumose stigmas, ovoid-oblong. Caryopsis, 2.5–3 mm long, ellipsoid, acute, sulcate on hilar side. Figure 13.

Distribution and Habitat

This species is endemic to the mountains of southern India. It is widely distributed in the Nilgiri Biosphere Reserve and is frequent in the understory of upper slopes of the hills above 1,800–2,400 m. In the Kerala region this species is located in the Palghat and Munnar forests (Seethalakshmi and Muktesh Kumar 1998; Muktesh Kumar 2011).

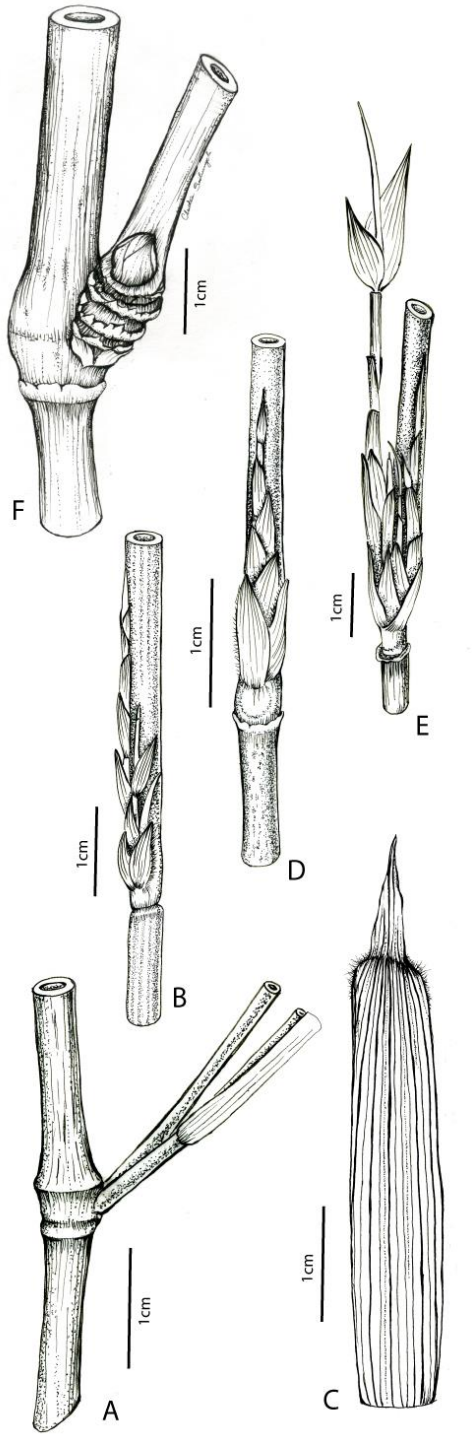
Phenology

This species is reported to flower annually without dying after flowering (Tewari 1992; Seethalakshmi and Muktesh Kumar, 1998). However, based on the specimens that we have seen from the Nilgiri district, a flowering specimen was collected in 1889, with vegetative specimens from 1883 and 1978, suggesting either a long flowering cycle or, more likely, insufficient herbarium records to infer flowering frequency and behavior.

Comments

This species possesses a set of characters that exclude it from Arundinaria s.s. For example, Arundinaria s.s. has leptomorph culm bases, glabrous culm leaves, usually well-developed auricles and poorly developed or absent girdles while K. wightiana possesses pachymorph culm bases, hispid culm leaves with golden brown irritating hairs, auricles absent and girdles present as a band at least 1 mm wide. The morphology of K. wightiana is consistent with Kuruna, so we here make the formal transfer. Within Kuruna, morphological comparison of K. wightiana with K. debilis and K. floribunda from Sri Lanka shows some resemblance among the three species based on the presence of a well-developed supranodal ridge, abaxially hispid culm leaves, and fimbriate culm leaf and foliage leaf sheath summits. The irritating golden brown hairs on the abaxial surface of the culm leaf sheath, the scaberulous culm internodes, and consistently 3 stigmas differentiate this species from the rest of *Kuruna*. So far, this is the only *Kuruna* species that is endemic to India, although a thorough investigation of Indian temperate woody bamboos might reveal additional endemic taxa. Further, though the inclusion of the south Indian temperate woody bamboo *Arundinaria wightiana* in *Kuruna* is based only on morphology, the close geographical proximity of the Western Ghats of India (where this

FIG. 13. *Kuruna wightiana*: A. Culm leaf. B. Flowering branch. C. Intermediate branch complement. D. Mature branch complement. E. Foliage leaf summit. F. Foliage leaf complement. G. Spikelet. H. Lemma. I. Palea. (Illustrations by C. Sidorowych, D based on *Soderstrom 2541*, E–F based on *Gamble 13359*, all others based on *Gamble 20733*).



species occurs) to Sri Lanka and its similar habitat to other species of the genus, support the decision to transfer *Arundinaria wightiana* to *Kuruna*. However, this species needs further study in a molecular analysis.

IUCN Red List category

Since *K. wightiana* was not collected in southern India during 2010 and authors have not seen this species in wild, we suggest the *Data Deficient (DD)* category for this species.

Additional Specimens Examined

INDIA: Kodaikanal taluk Kodai-Berijam road, fire tower, 2,400 m, 17 Dec 1989 (fl), *Periyanayagam 53985* (K); Nilgiri district, 2,134 m, Nov 1883, *Gamble 13359* (K); Nilgiri district, 2,438 m, May 1889 (fl), *Gamble 20733* (K); Nilgiri district, 2,286 m, Jan 1889 (fl), *Gamble 20332* (K); Nilgiri hills, near Ootacamund on

FIG. 14. A. *Kuruna floribunda*, distinct supranodal ridge. B. *K. densifolia*, early stage of branch complement. C. *K. densifolia*, culm leaf. D. *K. walkeriana*, early stage of branch complement. E. *K. scandens*, early stage of branch complement. F. *K. debilis* early stage of branching. (Illustrations by C. Sidorowych, A based on *Attigala et al. 139*, B–C based on *Attigala et al. 129*, D based on *Attigala et al. 162*, E based on *Attigala et al. 166* and F based on *Attigala et al. 124*).

road towards Naduvattam, Tamil Nadu, 17 Sep 1978, *Soderstrom 2541* (K).

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CHAPTER 4

PHYLOGENETIC RECONSTRUCTION OF ARUNDINARIEAE (BAMBUSOIDEAE: POACEAE) BASED ON PLASTOME PHYLOGENOMIC AND LOW-COPY NUCLEAR GENE ANALYSES

A manuscript to be submitted to *Molecular Phylogenetics and Evolution*

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Highlights

- Some conflicting phylogenetic signals from the plastome and low-copy nuclear gene phylogenies.
- Plastome phylogeny resolved all twelve major lineages within Arundinarieae.

- Low-copy nuclear gene phylogeny revealed Clade XII (*Kuruna*) as the early diverging lineage of Arundinarieae and a sister relationship between the clades IV (*Shibataea*) and V (*Phyllostacys*).
- Pachymorph rhizomes could be the ancestral condition while leptomorph rhizomes probably independently evolved multiple times with in Arundinarieae. The pseudospikelets may have evolved independently a minimum of two times during the evolutionary history of Arundinarieae, but possibly multiple times.

Abstract

We explore the phylogenetic relationships between the twelve major lineages of temperate woody bamboo clades (tribe: Arundinarieae) based on both plastomes and 3 low-copy nuclear gene sequence data. A representative sample of 28 taxa was used for the plastome sequence data phylogeny and 33 taxa were used for the low-copy nuclear marker phylogeny. Maximum parsimony, maximum likelihood and Bayesian inference analyses were conducted to reconstruct the Arundinaireae phylogeny based on both data sets. Most of the previously recognized major clades were supported by both data sets, though there were some conflicting phylogenetic signals. Plastome data revealed all twelve major clades within Arundinarieae. The low-copy nuclear gene phylogeny resolved most of the previously recognized major lineages of Arundinarieae such as clades I, II, III, VI, VII and XII except for clades IV and V. Further, low-copy nuclear gene phylogeny revealed Clade XII as the early diverging lineage of Arundinarieae and a sister relationship between the clades IV and V. The conflicting signals were further tested by taxon removal experiments, alternative hypothesis testing and Neighbor-Net analyses and depicted some difficult and complex relationships among these taxa mainly

due to recent hybridization and incomplete lineage sorting. Analyses of morphological character evolution of rhizomes and reproductive structures revealed that, pachymorph rhizomes could be the ancestral state while the leptomorph rhizomes probably evolved multiple times in Arundinarieae and also in Bambusoideae. Further, the pseudospikelets evolved independently multiple times during the evolution of Arundinarieae.

Keywords: Morphological character mapping, low-copy nuclear genes, *pabp1*, plastomes, pseudospikelets, *pvcel1*, rhizomes, *rpb2*.

Introduction

The subfamily Bambusoideae (Poaceae) includes mainly forest grasses that comprise 115 genera and approximately 1480 species (Bamboo Phylogeny Group [BPG], 2012; Clark et al., 2015). Bambusoideae is classified into two tribes of woody bamboos (the tropical Bambuseae and the temperate Arundinarieae) and one tribe of herbaceous bamboos (the Olyreae). Their distribution includes all continents except Antarctica and Europe (Clark et al., 2015; Wysocki et al., 2015). The temperate woody bamboos (Arundinarieae) are a diverse clade of 31 genera and ca. 546 species distributed primarily in forests of the northern temperate zone, but are also found at high elevations in tropical regions (Asia, high elevation habitats of Africa, Madagascar, India and Sri Lanka) (Clark et al., 2015). These temperate woody bamboo species are recognized by the presence of leptomorph, monopodial rhizomes (pachymorph in some species), basipetal vegetative branch development and tetraploidy (2n=48) (BPG, 2012; Clark et al., 2015). Even though unequivocal morphological synapomorphies have yet to be identified, monophyly

of the temperate woody bamboos is strongly supported by many molecular studies (BPG, 2012; Kelchner et al., 2013; Attigala et al., 2014).

Arundinarieae is well known for its complex taxonomy (Triplett et al., 2014). To date, there are twelve major lineages found in Arundinarieae based on chloroplast DNA (cpDNA), nuclear DNA genes and plastome analyses, but the relationships among these clades are still poorly understood (Triplett and Clark, 2010; Zeng et al., 2010; Yang et al., 2013; Attigala et al., 2014; Ma et al., 2014). Over the last few years full plastome phylogenomic techniques were used to resolve some of the evolutionary questions in Bambusoideae. Some studies used full plastome phylogenomic approaches to understand the subfamily level relationships and revealed that subfamilies Bambusoideae and Pooideae are more closely related than Ehrhartoideae (Zhang et al., 2011; Wu and Ge, 2012),while another study suggested that Bambusoideae is sister to the Erhartoideae and Pooideae (Wu et al., 2009). Wysocki et al. (2015) used a phylogenomic approach to understand the tribal level questions in Bambusoideae and found that Arundinarieae is sister to Olyreae and Bambuseae. Some authors used this approach to examine biogeographical questions especially focusing on *Arundinaria* (*Arundinaria applachiana*, *A. gigantea* and *A. tecta*) clade of Arundinarieae and *Crytpochloa strictiflora* and *Olyra latifolia* of Olyreae (Burke et al., 2012; Burke et al., 2014). They suggested that these new world bamboos accumulated and maintained unique plastome features over time and contributed to biogeogeraphic isolation from old world taxa. Further, their divergence estimates correlated with the major historic climatic changes.

Low copy nuclear genes reveal insights complementary to those from cpDNA and highly repetitive nuclear rDNA arrays such as ITS (Internal Transcribed Spacer). Recent

studies suggest that low copy nuclear genes can be successfully utilized in bamboos (Guo and Li, 2004; Yang et al., 2007; Peng et al., 2008; Yang et al., 2008; Yang et al., 2010; Zhang and al, 2012; Yang et al., 2013; Triplett et al., 2014; A. Fisher, pers. comm.). Several studies based on nuclear genes granule-bound starch synthase (GBSSI) and LEAFY showed poorly resolved relationships among the Arundinarieae clades (Zhang et al., 2012; Yang et al., 2013). Another recent study identified two ancestral genomes (A and B) due to hybridization and polyploidization in Arundinarieae based on low-copy nuclear genes (Triplett et al., 2014).

Prior to the advent of molecular phylogenetics, traditional classification schemes for the Arundinarieae (whether recognized as a tribe or not) used a combination of reproductive (i.e., conventional spikelets vs. pseudospikelets) and rhizome morphology (leptomorph vs. pachymorph) as well as stamen number to differentiate temperate woody bamboo genera and to classify them within subtribes (Keng, 1959, 1982a, 1982b, 1983a, 1983b; McClure 1966; Zhang, 1992). More recent non-molecular classifications (e.g., Soderstrom and Ellis, 1987) also incorporated anatomical or micromorphological characters. Based on rhizome types and reproductive structures, the taxa within currently recognized Arundinarieae were traditionally classified into three subtribes, the Arundinariinae, Shibataeinae and Thamnocalaminae (Zhang, 1992; BPG, 2012, Clark et al., 2015). However, several molecular studies unambiguously demonstrated the polyphyly of all three subtribes and thus caused the traditional classifications to be disregarded (Triplett and Clark, 2010; Zeng et al., 2010).

The primary objectives of the current study were to (1) understand the relationships among the Arundinarieae clades based on complete plastome sequences and

low-copy nuclear genes and (2) examine the evolution of some of the important morphological characters, rhizomes and reproductive structures.

Materials and Methods

Nuclear DNA gene selection

Three low copy nuclear genes: *cellulase1* (*pvcel1*), *poly-A binding protein1* (*pabp1*) and *RNA polymerase II* (*rpb2*), which encodes the second largest subunit of RNA polymerase II, were selected based on their level of variation and ease of amplification. All these loci have been used previously in phylogenetic studies of grasses: *pvcel1* was used in studies of *Panicum* (Triplett et al., 2012) and Bambusoideae (Triplett et al., 2014), *pabp1* has been used as a phylogenetic marker in *Oryza* (Zhu and Ge, 2005), *Panicum* (Triplett et al., 2012) and Bambusoideae (Triplett et al., 2014), and *rpb2* was used in a study of *Elymus* (Sun et al., 2007).

Taxon sampling and outgroup selection

Twenty eight species were selected for phylogenomic analysis, representing all twelve major clades in the Arundinarieae. 17 complete plastome sequences were downloaded from NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and 11 plastomes were newly generated during the current study. Two Pooid grasses: *Hordeum vulgare* and *Lolium perenne*; two herbaceous bamboos: *Cryptochloa strictiflora* and *Olyra latifolia*; two neotropical woody bamboos: *Chusquea spectabilis* and *Olmeca reflexa*; and two paleotropical woody bamboos: *Bambusa bambos* and *Melocanna baccifera* were selected as outgroups for the phylogenomic study (Supplementary material: Appendix A).

Thirty-three species were selected for the nuclear gene analyses. Forty-one *pabp1*and 46 *pvcel1* sequences were downloaded from NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Twenty five and 20 sequences were newly generated for *pabp1and pvcel1* respectively and 15 *rpb2* sequences were also newly generated during this study. Six species were selected as outgroups: *Brachyelytrum erectum* (Pooideae), *Chusquea bambusoides* (neotropical woody bamboos), *Chusquea spectabilis* (neotropical woody bamboos), *Chusquea scandens* (neotropical woody bamboos), *Guadua angustifolia* (neotropical woody bamboos), *Olyra latifolia* (Herbaceous bamboos) (Supplementary material: Appendix B).

DNA extraction, amplification, cloning and sequencing for nuclear gene analyses

Total genomic DNA was extracted from silica gel-dried leaves by Iowa State University DNA Facility's Autogenprep 740 DNA extraction robot. The primers used for PCR and sequencing reactions are listed in Table 1. All PCR and cycle-sequencing reactions were performed in Mastercycler® nexus (Eppendorf, Hamburg, Germany) thermal cyclers. PCR reactions were conducted in a 25 μl volume of Taq polymerase buffer, 100–500 ng total genomic DNA, 2.0mM MgCl2 0.4mM of both forward and reverse primers, 1.00mM dNTPs (0.25mM each dNTP), and two units of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA).

Table1. Low- copy nuclear DNA primers and PCR parameters used for amplification and sequencing.

Region	Primer Sequences (5' to 3')	PCR Parameters	Reference
pabp1	pabp1_for: GCTTGTCCGTAGAAGAGTTG pabp1_rev2:GTGTTAGCAAAGGGTCTGG ATTT	95^0C, 5m; 35x (95^0C, 30s; 49^0C, 45s; 72^0C, 1m 20s); 72^0C, 15m	Triplett et al., 2014
pvcel1	pvcel1_for: GCCAACATGGTTCAGTTGG pvcel1_rev: CGCCCCTCTGTGGTGTAC	95^0C, 5m; 35x (95^0C, 30s; 49^0C, 45s; 72^0C, 1m 20s); 72^0C, 15m	Triplett et al., 2014
rpb2	Bamboo 6F: CCAAATAGGGAGAAYACTATGG Bamboo 11aR: GTGAATCTTGTCATCMACCATATGC	95^0C, 3 m; 35x(95C, 1 m; 55^0C ,1 m; 72^0C, 1 m + 1 sec/cycle); 72^0C, 5 m	Current study (designed by Amy Denton)

PCR products were purified by agarose gel electrophoresis using EGel® CloneWell 0.8% SYBR® Safe gels with E-Gel® iBase™ Power System (Invitrogen) and cloned using the TOPO TA Cloning Kit (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol, except that all reaction volumes were quartered. To assess PCR errors and allelic sequences, 8 colonies were selected from each accession. Transformed colonies were used for PCR with primers M13 F-20 (GTA AAA CGA CGG CCA G) and M13 R (CAG GAA ACA GCT ATG AC), Exo-Sap method was used to clean the product (Dugan et al., 2002), and sequenced with region specific PCR primers following the ABI-Prism Big Dye Terminator sequencing method (version 3.1; Applied Biosystems, Foster City, CA, USA). Sequencing was performed on an ABI 3730xl DNA Analyzer (Perkin-Elmer, Applied Biosystems Division, Norwalk, Connecticut) by the DNA Sequencing Facility at Iowa State University.

DNA extraction, Illumina sequencing and quality control for phylogenomics

Genomic DNA was extracted for 11 bamboo species (*Bergbambos tessellata*, *Chimonocalamus* sp., *Fargesia nitida*, *Kuruna debilis*, *K. densifolia*, *Melocanna baccifera*, *Oldeania alpina*, *Pleioblastus hindsii* sensu Nakai, *Sasa veitchii*, *Shibataea kumasaca*, *Thamnocalamus spathiflorus*) from silica dried leaf tissues using the DNA extraction protocol in Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA).

Initial total genomic DNA concentrations were measured using the Qubit fluorometric quantitation system (Life Technologies, Grand Island, NY, USA) and were diluted to 2.5 ng/µl in 20 µl water. The Nextera Illumina library preparation kit (Illumina, San Diego, CA, USA) was used to prepare libraries for sequencing and the DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) was used for library sample purification. Sequencing was performed on a HiSeq 2000 (Illumina, San Diego, CA, USA) using single reads at the Iowa State University DNA Sequencing Facility, Ames, IA, USA. The reads generated by this method were 100 bp in length.

SolexaQA software package's DynamicTrim v2.1 (Cox et al., 2010) was used with default settings to perform the initial quality filtering of all reads and then the short fragments [< 25 bp in length (default setting)] were removed with LengthSort v2.1 in the same package.

Assembly and alignment of plastomes

A *de novo* method was used to assemble the entire plastomes. The Velvet software package was run iteratively similar to methods described in Wysocki et al. (2014). Any remaining gaps in the plastomes were determined using contigs or reads by locating overlapping regions of at least 20 bp until the circular map was complete with no

gaps or ambiguities. All 28 genome sequences were aligned with MAFFT V7.017 (Katoh et al., 2005). The alignments were checked manually and the poorly aligned regions were adjusted. Autapomorphic, parsimony uninformative characters were excluded prior to analysis.

Treatment and concatenation strategy, alignment and gap coding of low-copy nuclear gene sequences

Ambiguous bases and vector sequences were removed from the ends of both forward and reverse reads manually. Clone sequences were imported and manually inspected with GENEIOUS R7 (Biomatters Ltd., Auckland, NZ). Ambiguous bases in each clone sequence were corrected manually by comparing sequence quality from trace files. Corrected clones were assembled into accession-specific files and aligned with GENEIOUS R7 (Biomatters Ltd., Auckland, NZ). PCR mediated recombinants (chimeras) were excluded by eye through careful inspection of the alignment as these recombinant sequences can increase homoplasy and misrepresent the true phylogeny. Consensus sequences for each sequence type per species were obtained in order to minimize the inclusion of sequencing errors. Generally, a substitution that appeared in a single sequence was considered as PCR error and sequences with two or more nucleotide differences were considered as different alleles. The sequences were considered to represent different homeologs only if they clustered in different major clades and were then labelled as A or B. Autapomorphic, parsimony uninformative indels were not scored, and they were excluded along with other gaps prior to analysis.

Phylogenetic and phylogenomic analyses of Arundinarieae

A and B homeologs of nuclear genes were considered as independent datasets for phylogenetic analyses as no recombination was detected among the two copies for the three gene (Griffin et al., 2011). Hence, they were treated as separately-evolving gene copies. Only the A copy of *rpb2* was used due to amplification difficulties. Five datasets (A and B homeologs of *pvcel1*, A and B homeolgs of *pabp1* and A homeologs of *rpb2*) were used for phylogenetic analyses of nuclear genes. Separate analyses were performed for each locus first to see if similar results were obtained regardless of search criterion. A combined dataset analysis was also performed.

For the different datasets: the complete plastomes, each of the three low-copy nuclear regions and the combined nuclear dataset (*pabp1*-A homeolog + *pabp1*-B homeolog + *pvcel1*-A homeolog + *pcvel11*-B homeolog + *rpb2* -A homeolog) were all analyzed using: maximum parsimony (MP) with PAUP* 4.0b10 (Swofford, 2002), maximum likelihood (ML) with RAxML version 8.0.X (Stamatakis, 2006) and Bayesian inference (BI) with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2003; Ronquist et al., 2011). MP analyses were performed with 1000 random addition sequences, tree bisection-reconnection (TBR) and branch swapping. A full heuristic bootstrap was conducted for MP with 1000 bootstrap replicates to assess the relative support for each node. Akaike information criterion (AIC) calculations, implemented in JmodelTest 0.1 (Guindon and Gascuel, 2003; Posada, 2008), were used to select the appropriate model of sequence evolution for the plastome dataset. The general time-reversible model of DNA sequence evolution with invariant sites and gamma-distributed rate heterogeneity (GTR+I+G) was among a group of equally best fit models (found in

the 100% confidence intervals). Hence the GTR+I+G model was used in ML analysis. ML analyses were conducted by rapid bootstrap (1000 replicates) analysis and searching for the best-scoring ML Tree with the GTR+I+G model. BI was conducted with flat priors. The Markov chain Monte-Carlo algorithm was executed for four chains for 10,000,000 generations per run, sampling every 2,000 generations, and a chain-heating temperature of 0.2; this entire procedure was conducted twice. Posterior Probabilities (PP) were analyzed after a burn-in of 1,000,000 trees and then the remaining samples were summarized and a majority-rule consensus tree was constructed. When assessing conflicts among the resultant phylogenies, the threshold value for the bootstrap criterion for both MP and ML was 70% and posterior probability measure for BI was 0.95 (Mason-Gamer and Kellogg 1996, Wilcox et al. 2002). Values less than 70% MP Bootstrap (MPBS)/ML Bootstrap (MLBS) and less than 0.95 PP were considered as lacking support. If MPBS and MLBS were above 95% and BI was 1.00 that was considered as strong support. It was considered moderate support if the MPBS and MLBS were 95% - 85% and BI was 0.99. If the MPBS and MLBS were 70% - 85% and BI was 0.95 - 0.99, then it was considered as low level of support.

Testing for long-branch attraction (LBA)

To test if there were any potential errors caused by LBA, taxon removal experiments were performed (Kelchner et al., 2013). Especially to test if outgroup selection affected the topology of our ingroup, ML (model: GTR + I + G) analyses were conducted for both plastome and low-copy nuclear gene datasets. The following experiments were conducted individually by removing 1) only Pooideae taxa, 2) Pooideae and Olyreae taxa, 3) Pooideae and Bambuseae taxa, 4) Bambuseae and Olyreae

taxa, and 5) all outgroup taxa. The ML topology from each analysis was compared with the original plastome tree topology for changes in relationships among the remaining taxa.

Alternate hypothesis testing

We tested whether the plastome dataset provided sufficient evidence to reject particular hypotheses of relationships suggested by previous studies (e.g., monophyly of the *Arundinaria* clade plus the *Phyllostachys* clade, monophyly of the *Kuruna* clade plus *Bergbambos*, the sister relationship of the *Kuruna* clade to the rest of the temperate woody bamboo clades, etc.). Constraint trees were generated in MacClade 4.08 (Maddison and Maddison, 2005) by forcing test groups to be monophyletic or sister, but otherwise allowing taxa to "float," and ML analyses were performed in RAxML version 8.0.X (Stamatakis, 2006) using each constraint in turn. The Shimodaira- Hasegawa test (SH) test (Shimodaira and Hasegawa, 1999) as implemented in RAxML version 8.0.X was then used to test the significance of differences in tree statistics amongst different topologies in comparison with the ML plastome data tree topology.

A similar approach was followed to test some of the conflicting relationships between plastome and low-copy nuclear gene tree topologies and the alternative hypotheses were tested against the ML low-copy nuclear gene tree topology.

Network Analysis

As another means of visualizing the signal in the dataset and to evaluate possible phylogenetic signal conflicts, Neighbor-Net analysis was performed with SplitsTree4 v. 4.13.1 (Huson and Bryant, 2006) for each dataset separately for plastome sequences, *pvcel1* A homeologs and *pabp1* A homeologs. Due to a lower number of sequences for

the A homeologs of *rpb2*, a network analysis was not performed for this dataset. Preliminary analyses of both plastome and low-copy nuclear datasets revealed that removal of some taxa significantly reduced character conflicts. Thus, additional separate Neighbor-Net analyses were conducted excluding *Indocalamus sinicus* from the plastome dataset and the two *Sasa longiligulata* alleles from the *pabp1* dataset.

Morphological character mapping

A matrix of morphological character states was generated for each taxon for the characters: rhizome types and reproductive structures based on the herbarium specimens (ISC, K, MO, US) and published literature (Soderstrom and Ellis, 1988; Clayton et al., 2006 onwards; Flora of China, 2006; Attigala et al., 2014). The two forms of rhizome types seen in Bambusoideae are the leptomorph and pachymorh rhizomes (McClure, 1966). For the purpose of character state coding, leptomorph and pachymorph rhizomes were considered as 0 and 1 respectively without considering any tillering or neck length. The two main forms of reproductive structures seen in Bambusoideae are the conventional spikelets and pseudospikelets. A conventional grass spikelet bears two empty proximal glumes and one to many distal florets. However, pseudospikelets are complex spikelet structures. A typical pseudospikelet consists of a subtending bract, a prophyll, one or more gemmiparous bracts, 0 to a few empty bracts (glumes) and the spikelet proper (BPG, 2012; Tyrrell et al., 2012). Absence of pseudospikelets were scored as 0 while pseudospikelets lacking only subtending bracts were referred as incomplete pseudospikelets and scored as 1.The pseudospikelets with all four different structures were considered as complete pseudospikelets and scored as 2. (Supplementary material: Appendix C). With the use of "Trace character history" option, a simple ancestral

character state reconstruction was performed with the parsimony ancestral states method in the program Mesquite V 2.75 (Maddison and Maddison, 2011). Both rhizome types and reproductive structures were mapped on the phylogenetic tree topology obtained from the plastome data using the above method.

Results

New genome assembly and alignment of plastomes

Eleven temperate woody bamboo genomes were newly generated during the current study (Supplementary material: Appendix A). These newly synthesized plastomes varied from 139,441 to 139,731 base pairs (bp) in length.

The multiple sequence alignment of all 28 species (including both newly generated and NCBI Genbank downloaded plastomes) was 144,296 bp in length. Of the total aligned length, 87.83% of the sites were invariable. Further, of the variable characters 17,566 (12.17%) only 6,325 (4.38%) characters were parsimony informative.

Phylogenomic analysis

The MP analysis of the plastome dataset resulted a single most parsimonious tree with a length of 22,493 steps, consistency index (CI) 0.8566 and Homoplasy index (HI) 0.1434. The MP tree topology was mostly similar to the ML and BI tree topologies of the plastome dataset, but with few exceptions. Fig.1 shows the phylogeny obtained from BI with all three different support values: MP bootstraps (MPBS), ML bootstraps (MLBS) and the BI posterior probabilities (PP). Unlike the MP phylogeny, both ML and BI tree topologies were identical to each other with differing support values.

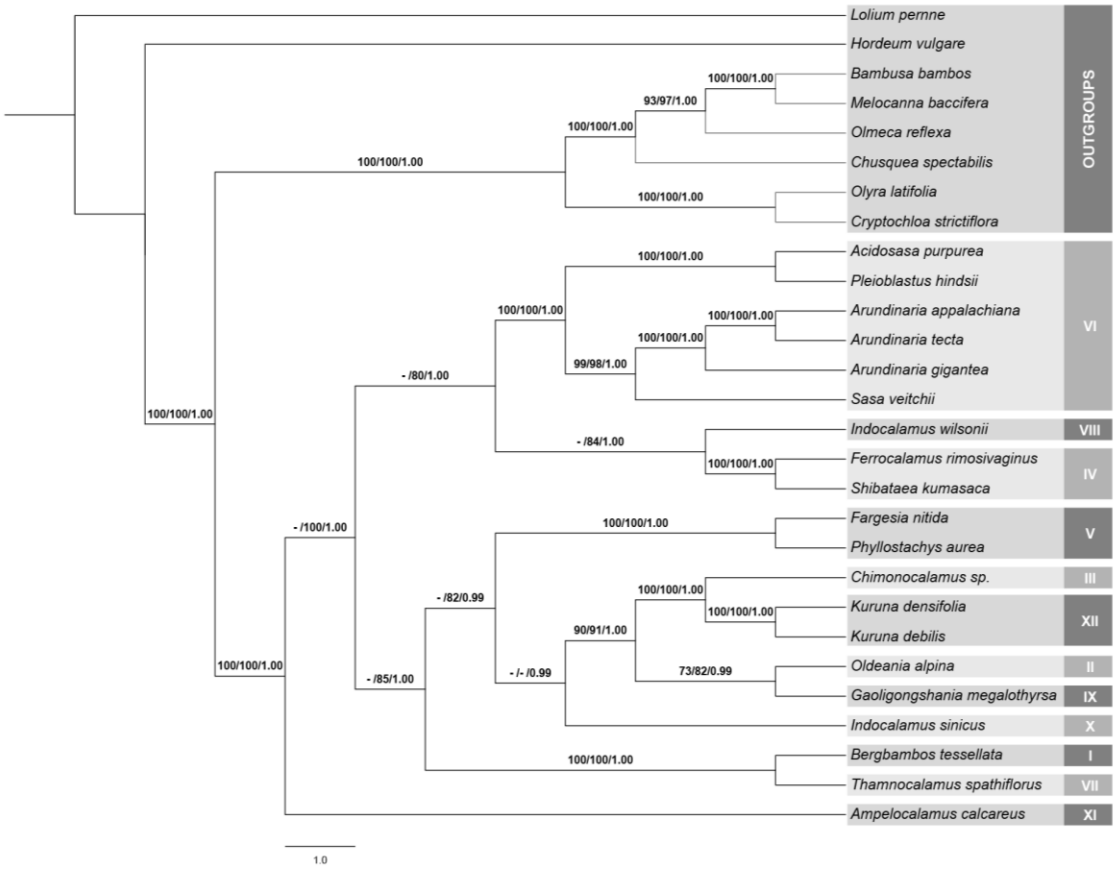

Fig. 1. Phylogenetic estimation of Arundinarieae based on plastome sequences inferred from 28 taxa representing all 12 major lineages of the tribe. Numbers indicate bootstrap values ≥ 70% from MP and ML analyses and posterior probabilities ≥ 0.95 from the BI analyses, respectively. All values below these threshold support values were indicated with a "-". Roman numerals and the shadings associated with each clade represent the 12 different lineages of Arundinarieae.

All three analyses strongly supported the monophyly of Bambusoideae and Arundinarieae. The sister relationship of Arundinarieae to the rest of the Bambusoideae (Bambuseae plus Olyreae) and the sister relationship between Bambuseae and Olyreae was robustly supported with 100% MPBS, 100% MLBS and 1.00 PP. As seen in Fig. 1, MP, ML and BI analyses of the plastomes recovered all major 12 temperate woody bamboo lineages: *Bergbambos* (Clade I), African alpine bamboos (Clade II), *Chimonocalamus* (Clade III), *Shibataea* clade (Clade IV), *Phyllostachys* clade (Clade V), *Arundinaria* clade (Clade VI), *Thamnocalamus* (Clade VII), *Indocalamus wilsonii* (Clade

VIII), *Gaoligongshania* (Clade IX), *Indocalamus sinicus* (Clade X), *Ampelocalamus calcareus* (Clade XI) and the *Kuruna* clade (Clade XII). According to all three analyses *Ampelocalamus calcareus* (Clade XI) was well supported as the sister to the rest of the temperate woody bamboo clades. Of the 19 total ingroup nodes, 10 nodes were strongly supported (100% MPBS, 100% MLBS, 1.00 PP) and only 3 nodes were moderately supported in all the 3 analyses. Furthermore, one node received strong support (100% MLBS, 1.00 PP) only from ML and BI analyses. This is the node that clustered clades I, II, III, V, VII, XII and clades IV, VI, VIII into two separate groups. The remaining six nodes were moderately supported only in ML and BI analyses and these nodes were not resolved in the MP analysis. In addition, there was significant evidence (100% MPBS, 100% MLBS, 1.00 PP) to support the sister relationship of the *Kuruna* + *Chimonocalamus* clade and the *Bergbambos* + *Thamnocalamus* clade in all analyses.

Phylogenetics based on low-copy nuclear genes

Fig. 2 shows the BI phylogenetic tree with the support values from all three analyses summarized for the dataset with all 3 low-copy nuclear genes combined. The MP analysis resulted in the most parsimonious tree (1883 steps, 0.8317 CI and 0.1683 HI). Of the 5,944 characters only 547 were parsimony informative. Like the plastome data results, the MP analysis of the combined low-copy nuclear datasets showed a tree topology mostly congruent to the MP and BI analyses with few exceptions, while MP and BI analyses resulted identical tree topologies. The low-copy nuclear dataset represented only eight clades (Clades I, II, III, IV, V, VI, VII and XII) of the twelve major lineages of Arundinarieae. Of the eight represented clades, six were well resolved with high support in all three analyses (Clades I, II, IV, V, VII and XII). However, the *Chimonocalamus*

clade (Clade III) was well supported only in the BI analysis and this clade did not include *Chimonocalamus delicatus*. The *Thamnocalamus* clade (Clade VII) grouped with *Ferrocalamus rimosivaginus* and this relationship was supported only in the BI analysis.

The sister relationship between the D copy of Bambuseae and the H copy of Olyreae was strongly supported by ML (96% MLBS) and BI (1.00 PP) while MP provided moderate support (82% MPBS). A sister relationship was strongly evident for the clade consisting of the D copy of Bambuseae + the H copy of Olyreae, to the rest of the taxa including the C copy of Bambuseae and Arundinarieae from all three analyses (99% MPBS; 100% MLBS; 1.00 PP).Within Arundinarieae, the *Shibataea* + *Phyllostachys* clade was monophyletic with strong support (99% MPBS; 100% MLBS; 1.00 PP) and this *Shibataea* + *Phyllostachys* clade was sister to the *Arundinaria* clade (Clade VI) with 92% MLBS, 1.00 PP and 79% MPBS support. In addition, there was high support for the sister relationship of the *Kuruna* clade to the rest of the Arundinarieae taxa (99% MPBS; 98% MLBS; 1.00 PP). However, the relationships among the other clades (Clades I, II III and VII) were poorly supported. A few taxa were "incorrectly" positioned in the low-copy nuclear tree topology relative to the plastome topology such as the sister relationship of *Sasa longiligulata* (belongs to Clade IV), the clustering of *Yushania niitakayamensis* (belongs to Clade V) with the African alpine species; *Fargesia nitida* (belongs to Clade V) with the *Chimonocalamus* clade; and *Ferrocalamus rimosivaginus* (belongs to Clade IV) with the *Thamnocalamus* clade.

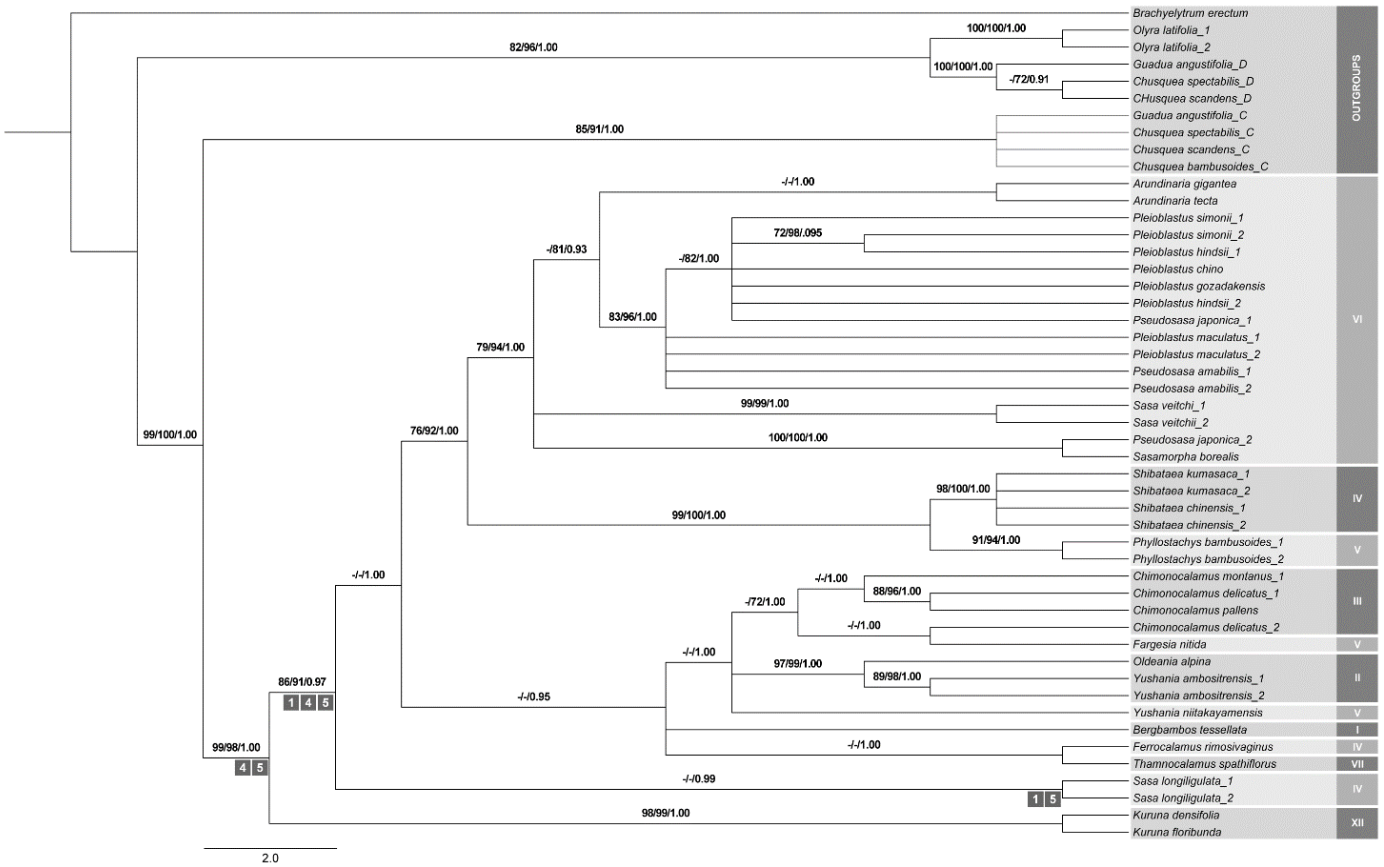

Fig. 2. Phylogenetic estimation of Arundinarieae based on 3 low- copy nuclear genes (*pabp1*, *pvcel1* and *rpb2*) treating the A and B homeologs as independent datasets. Numbers above the node indicate bootstrap values ≥ 70% from MP and ML analyses and posterior probabilities ≥ 0.95 from the BI analyses, respectively. All values below these threshold support values were indicated with a "-". Roman numerals and the shadings associated with each clade represent the 12 different lineages of Arundinarieae. The numbers in gray boxes below the nodes indicate the taxon removal experiments (conducted to infer long-branch attraction) that did not support that particular relationship/node; 1: only Pooideae taxa removed, 4: only Bambuseae and Olyreae taxa removed, 5: all outgroup taxa removed.

LBA and alternative hypothesis testing

All five taxon removal experiments performed to test for potential LBA for the plastome dataset resulted in tree topologies identical to the original plastome tree topology regardless of the outgroup taxa (topologies not shown). Conversely, some of the taxon removal experiments conducted for the low-copy nuclear dataset resulted in different tree topologies. For the low-copy nuclear gene dataset, the tests that removed only Pooideae taxa, only Bambuseae and Olyreae taxa, or all outgroup taxa generated a different topology than the phylogenetic tree estimation based on all 33 taxa. However, the other two taxon removal experiments, which removed only Pooideae and Olyreae taxa or only Pooideae and Bambuseae taxa, resulted in tree topologies identical to the original low-copy nuclear gene phylogenetic tree estimation based on all 33 taxa (Fig. 2).

Results of the SH test for plastome and low-copy nuclear datasets are summarized in Table 2. Based on the SH test for plastomes, our data reject the monophyly of following: clades Clades V + VII (*Phyllostachys* + *Thamnocalamus*), Clades I + XII (*Bergbambos* + *Kuruna*), Clades VII + XII (*Thamnocalamus* + *Kuruna*), Clades I + II + (*Bergbambos* + African alpine bamboos) and Clades XII + VII + I + II (*Kuruna* + *Thamnocalamus* + *Bergbambos* + African alpine bamboos). Our data also reject the sister relationship of the *Kuruna* clade (Clade XII) to the remaining Arundinarieae. Thus, all these alternative hypotheses generated significantly worse compared to the original ML tree topology.

Table 2. Results of Shimodaira-Hasegawa (SH) test implemented in RAxML for the Plastome dataset (*) and for the low-copy nuclear dataset (▲). D (LH) is the difference in log likelihood units between the best constrained tree and the best unconstrained tree. SD: Standard deviation.

Topological constraints (Alternative hypotheses)	**Likelihood[1]**	**D (LH)**	**SD**	**Significantly worse ($p < 0.01$)**
*Clade V + Clade VII monophyletic	-342760.8306	-1160.8234	83.7892	Yes
*Clade XII sister to the rest of the temperate woody bamboos	-341981.1900	-381.1738	47.2489	Yes
*Clade XII + Clade I monophyletic	-342743.3052	-1143.2980	82.6732	Yes
*Clade XII + Clade VII monophyletic	-342748.6272	-1148.6201	82.5431	Yes
*Clade I + Clade II monophyletic	-342786.1484	-1186.1412	81.9190	Yes
*Clade XII + VII + I + II monophyletic	-341861.3827	-261.3754	43.1728	Yes
▲Clade I + Clade VII monophyletic	-19015.0407	-14.1150	19.0989	**No**
▲Clade VI + Clade IV monophyletic	-19029.9841	-29.0584	12.0027	**No**
▲Clade II + Clade III + Clade XII monophyletic	-19102.0273	-101.1015	25.3188	Yes
▲Clade II + Clade III + Clade V + Clade XII monophyletic	-19177.0317	-176.1059	34.4745	Yes
▲*Sasa longiligulata* in Clade IV	-19089.1231	-88.1973	19.8965	Yes
▲*Ferrocalamus rimosivaginus* in Clade IV	-19058.4610	-57.5353	24.2835	**No**
▲Both *Sasa longiligulata* and *Ferrocalamus rimosivaginus* in Clade IV	-19110.2974	-109.3716	27.4378	Yes
▲*Yushania niittakayamensis* in Clade V	-19053.5619	-52.6362	18.4938	Yes
▲*Fargesia nitida* in Clade V	-19087.5694	-86.6437	36.0886	**No**
▲Both *Yushania niittakayamensis* and *Fargesia nitida* in Clade V	-19101.4536	-100.5279	30.0938	Yes

The SH tests for the low-copy nuclear dataset could not reject the following alternative hypotheses: the monophyly of Clade I + Clade II (*Bergbamos* + *Thamnocalamus*); Clade VI + Clade IV (*Arundinaria* + *Shibataea*); clustering of *Ferrocalamus rimosivaginus* with Clade IV (*Shibataea*) and clustering of Fargesia nitida with Clade V (*Phyllostachys*). The other six alternative tree topologies were significantly worse than the original tree topology that was obtained for the low-copy nuclear genes.

3.5 Network analysis

The Neighbor-Net analysis of plastome mainly supported the twelve major clades with the exception of *Indocalamus sinicus* showing some character conflicts (Fig. 3A). However, with the exclusion of *Indocalamus sinicus* from the plastome dataset, a significantly improved network with lower character conflicts resulted and it supported the other eleven clades of Arundinarieae (Supplemental material Figure 1). Furthermore, some character conflicts were visible for *Acidosasa purpurea* in its placement between *Sasa veitchii* and *Pleioblastus hindsii* sensu Nakai. Except for these two above-mentioned character conflicts, the other relationships are tree-like and represented all the major clades in the tribe (Fig. 3A).

However, the Neighbor-Net analyses of low-copy nuclear genes *pabp1* and *pvcel1* revealed some conflicting signals with the plastome network as expected, though they agreed with the low-copy nuclear phylogenetic tree topology (Fig. 3B, C). With the exception of a few taxa not ending up within their corresponding clades (underlined taxa in Fig 3B and Fig 3C), the two networks of *pabp1* and *pvcel1*showed all eight clades represented here of the 12 major clades of Arundinarieae. Both *pvcel1* and *pabp1* revealed a highly reticulate, narrowly-meshed network between most of the *Arundinaria*

(Clade VI) and *Phyllostachys* (Clade V) clades. Similar results were obtained for the old world temperate clades such as the African alpine bamboos (Clade II), *Chimonocalamus* (Clade III) and *Kuruna* (Clade XII).

Morphological character mapping

Based on the morphological character mapping, pachymorph rhizomes are evident in Bambuseae, Olyreae and most of the major clade I+ II+ III+ V+ VI+ XII of Arundinarieae. However, some members in *Phyllostachys* clade (Clade V), *Indocalamus sinicus* (Clade X) possess leptomorph rhizomes. Unlike the rhizomes, no clear evolutionary pattern was observed in pseudospikelet development in the Arundinarieae. Among outgroup members, only the Paleotropical bamboos, namely members of Bambusinae (*Bambusa bambos*) and Melocanninae (*Melocanna baccifera*) show the presence of pseudospikelets. Within the Arundinarieae, only *Phyllostachys aurea* of the *Phyllostachys* clade and *Shibataea kumasaca* of the *Shibataea* clade possess pseudospikelets.

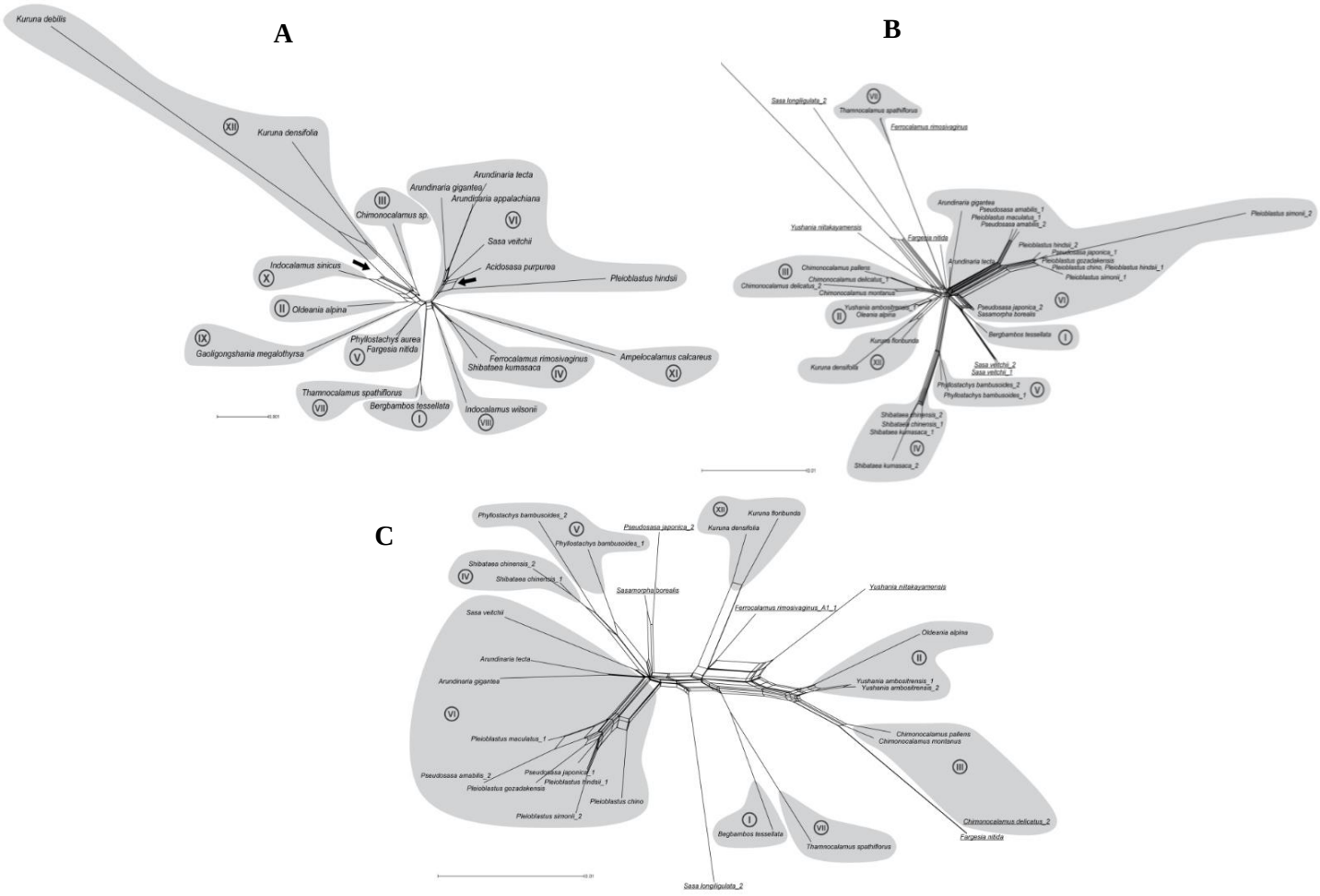

Fig. 3. Neighbor-Net analyses of Arundinarieae based on: A) Plastome data; B) *pvcel1* low-copy nuclear gene data; C) *pabp1* low-copy nuclear gene data. Roman numerals represent the major Arundinarieae clades. Arrows indicate the two major character conflicts seen in the plastome dataset.The underlined taxa are the ones that are placed out of their corresponding clades showing disagreements.

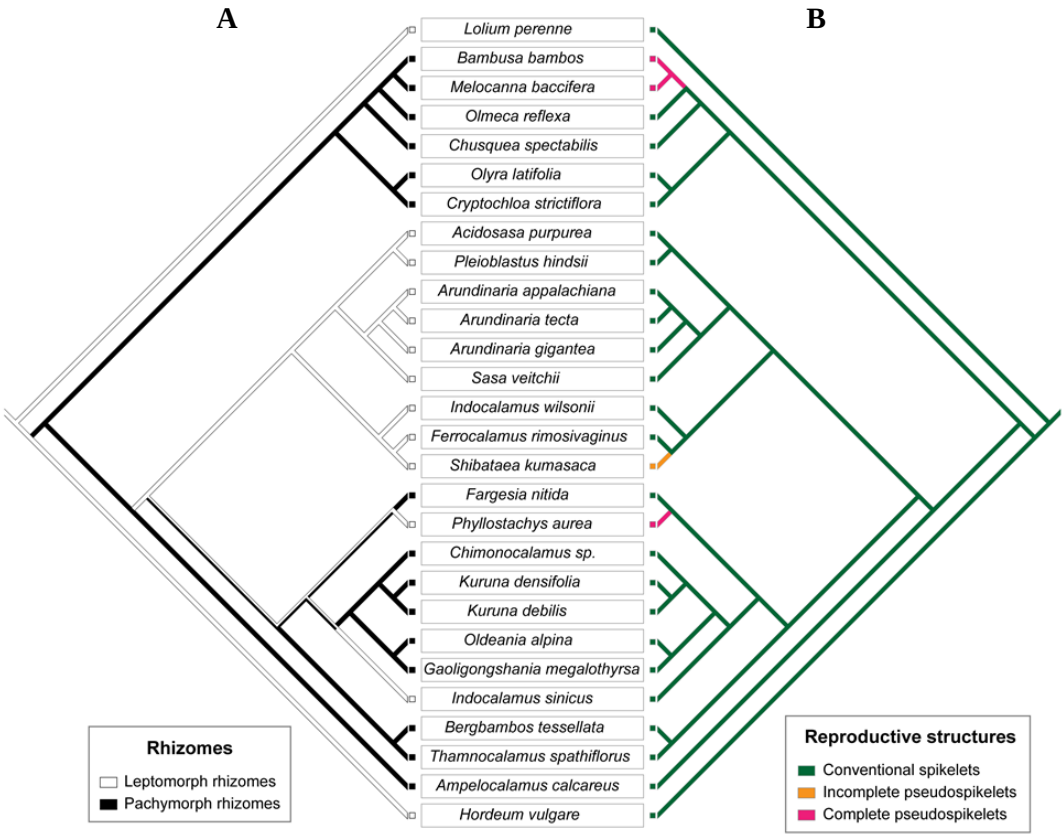

Fig. 4. Morphological character evolution mapped on to the plastome phylogenetic estimation. A. rhizome types; B. reproductive structures.

Discussion

Plastome phylogenomics

The newly synthesized plastome sequence lengths were very similar to previously published cp genomes of temperate woody bamboos (Yang et al., 2013; Ma et al., 2014; Wysocki et al., 2015). The 87.83% of the invariable sites indicates the low level of sequence variability in the temperate woody bamboo plastomes.

The MP phylogeny was mostly congruent with ML and BI phylogenies with few exceptions (Fig. 1) and could consider as a less resolved phylogeny compared to ML and

BI analyses. Monophyly of the subfamily Bambusoideae and the tribes Arundinarieae, Bambuseae and Olyreae retrieved in the current study confirm the strong support for these lineages identified in previous studies (Kelchner et al., 2013; Ma et al., 2014; Wysocki et al., 2015). Notably, the current study supports the paraphyly of the woody bamboo syndrome as noted in Wysocki et al. (2015). Based on the current plastome phylogeny it is suggestive that the woody habit independently evolved in Bambusoideae, in the tribe Bambuseae (Tropical woody bamboos) with another origin in the tribe Arundinarieae (temperate woody bamboo). However, the maternally inherited plastome provides only a part of the evolutionary signal and this is further discussed in the low-copy nuclear gene phylogeny analyses (See below).

A recent study (Ma et al., 2014) showed evolutionary relationships within Arundinarieae based on plastome phylogenomic studies. However, their whole chloroplast genome phylogeny included only eight of the 12 major lineages. The current study included all 12 major lineages of Arundinarieae and consistent with Ma et al. (2014), we also recovered Clade XI (*Ampelocalamus calcareus*) as the sister clade to the remaining Arundinarieae. Though Ma et al. (2014) recovered unresolved or contrasting branching order among Clades IV, VI and VIII, the current study revealed high support from BI and moderate support from ML analyses for the sister relationship of Clade IV + VIII and the sister relationship of Clade VI with Clade IV + VIII.

Current study has not shown any potential evidence for patterns of geographic origin of the clades. For example, none of the *Kuruna* clade species or Indian *Thamnoclamus* clade species formed a clade and no clade was formed between African alpine bamboo species and the *Bergbambos* clade. However, *Arundinaria* s.s., which

includes only species distributed in eastern North America, was well supported as monophyletic.

In addition, the well supported sister relationship between *Bergbambos tessellata* (Clade I) and *Thamnocalamus spathiflorus* (Clade VII) shows the molecular phylogenetic evidence for the previous classification of the *Bergbambos tessellata* as *Thamnocalamus tessellatus*.

Phylogenetics based on low-copy nuclear genes

Comparing the low-copy phylogeny to the plastome phylogeny, several similarities emerge. In both phylogenies, *Bergbambos*, the African alpine bamboos and *Chimonocalamus* resolve within one major group and also *Arundinaria* and *Shibataea* resolve into the other major group. Further, the nuclear phylogeny supports the sister relationship between Olyreae and Bambuseae as evident from the plastome phylogeny and several previous studies. In addition, some novel and unexpected results of the low-copy nuclear gene trees, are the position of the *Kuruna* clade as the early diverging lineage of Arundinarieae and the monophyly between the *Phyllostachys* and *Shibataea* clades.

As suggested by previous studies, there could be several reasons for these complex and incongruent results between the plastome and nuclear phylogenies of Arundinarieae, including intergeneric hybridization and reticulate evolution, rapid radiation, convergent evolution, and incomplete lineage sorting (Triplett and Clark 2010; Zeng et al. 2010; Zhang et al., 2012; Yang et al., 2013; Attigala et al., 2014; Ma et al., 2014; Triplett et al., 2014). Studies focused on artificially produced hybrids between several temperate woody bamboo genera suggest that their mating systems allow cross

pollination between different species of Arundinarieae, if there is an opportunity (Muramatsu, 1972a, 1972b; Hatakeyama et al., 1987; Suzuki, 1987; Okamura et al., 1991). A recent study discussed the hybridization in the temperate woody bamboo clade based on AFLP and cpDNA sequence data in detail and discovered hybrid origins for species in several widespread and well-known genera, including *Hibanobambusa*, *Sasaella*, and *Semiarundinaria* (Triplett and Clark, in revision). Thus, hybridization could be one of the major causes of these incongruent results between plastome and low-copy nuclear tree topologies, such as the contrasting placements of the *Shibataea* and *Phyllostachys* clades and the isolated position of *Sasa longiligulata* in the low-copy nuclear tree. Moreover, additional work is necessary to understand the evolutionary relationship of *Kuruna* within Arundinarieae with wider sampling including more representatives from South India and the high elevation temperate woody bamboos from Madagascar.

Species level phylogenies are often problematic with nucleotide sequence data when a plant group has undergone recent and rapid radiation (Baldwin and Sanderson, 1998; Richardson et al., 2001; Malcomber, 2002). Further, relatively recent and rapid diversification within Arundinarieae has been proposed as another cause of poor resolution of relationships among these clades especially at the generic level (Stapleton et al., 2009; Hodkinson et al., 2010; Stapleton, 2013), since a study (Bouchenak-Khelladi et al., 2010) estimated that the Arundinarieae is a relatively young lineage within Bambusoideae with an origin dating to ~15 mya. In addition, convergent evolution among distant lineages can produce morphological homoplasy that complicates phylogeny and could result incongruence. Further, incomplete lineage sorting can

possibly cause phylogenetic incongruence in many comparative genomics datasets. Population genetic theory of the coalescent states that sufficiently close speciation events will lead to incongruence due to incomplete lineage sorting (Maddison, 1997) and an ancestral polymorphism with subsequent lineage sorting could produce molecular or morphological signals that are difficult to distinguish from hybridization (Doyle et al., 1999; Avise, 2000; Sang and Zhong, 2000; Peters et al., 2007; Maureira-Butler et al., 2008) Therefore, as studies (Triplett and Clark, 2010; Yang et al., 2013; Ma et al., 2014) suggest, incomplete lineage sorting could be another possible explanation for the phylogenetic incongruence. There is also the likelihood of relatively low, but unavoidable stochastic and systematic errors (Kelchner, 2009) and thus additional thorough investigation of Arundinarieae relationships with many different low-copy nuclear markers is needed.

Testing LBA and alternative tree topologies

One of the most common manifestations of LBA is that distantly related outgroups have a biased attraction to long branches within the ingroups (Sullivan and Swofford, 1997; Lin et al., 2002; Graham et al., 2002; Bergsten, 2005). Thus a common suggestion is to conduct the phylogenetic analyses both with and without outgroups to compare whether the distantly related outgroups changes the ingroup topology. The taxon removal approach we used by outgroup removal resulted in no changes to the ingroup tree topology of the plastome data, indicating that the plastome tree topology probably was not affected by LBA. Nevertheless, the taxon removal experiments performed for the low-copy nuclear gene phylogeny did not support some nodes/relationships. *Kuruna* as the early diverging lineage, monophyly of the two alleles *of Sasa longiligulata* and the

sister relationship of *Sasa longiligulata* to the rest of the Arundinarieae were nor supported by some taxon removal experiments and could be due to LBA.

The alternative hypotheses for the plastome dataset that we tested were selected for various reasons. Ma et al. (2014) recovered moderate support from MP and ML (88% and 85% respectively) while PP support was high (1.00) for the monophyly of Clade V + Clade VII in their complete plastome phylogeny. Thus we decided to test this relationship with our plastome dataset. The hypothesis that the sister relationship of Clade XII to the rest of the Arundinarieae is due to a previous suggestion by Triplett and Clark (2010) that the Sri Lankan *Kuruna* species could be the early diverging lineage of Arundinarieae. In addition, a recent study of Arundinarieae based on five plastid markers could not reject the monophyly of Clade XII + Clade I (Attigala et al., 2014) and thus we tested it as an alternative hypothesis. Further, clade origin due to close geographical proximity was also tested, e.g., all the Sri Lankan and Indian taxa form a clade (Clade XII + Clade VII) or the African species form a clade (Clade I + Clade II). Though there are currently several clades in Arundinarieae that represent the Indian, Sri Lankan, African and Madagascan temperate woody bamboo species, traditionally these taxa were all classified in the currently recognized genus *Arundinaria*. Thus we tested the alternative hypothesis of the monophyly of Clades XII + VII + II + I.

We tested some of the relationships suggested by the plastome dataset as alternative hypotheses with the low-copy nuclear gene tree topology. Since the plastome analyses provided strong evidence for the monophyly of *Bergbambos* + *Thanmocalamus*; *Arundinaria* + *Shibataea*; the African alpine bamboos + *Chimonocalamus* + *Kuruna*; and the African alpine bamboos + *Chimonocalamus* + *Kuruna* + *Phyllostachys*, we tested

these hypotheses with the low-copy nuclear dataset. Of those, the first two were not significantly different ($p < 0.01$) from the original low-copy nuclear tree topology suggesting that these could be the actual relationships between these clades. Further, the other alternative hypotheses in which we forced some of the taxa to be in their clades as determined by plastome data, e.g., *Ferrocalamus rimosivaginus* in Clade IV and *Fargesia nitida* in Clade V, were not rejected by the SH test. This also suggests that these proposed relationships could be correct.

Network analysis

The Neighbor-Net analyses agreed with the tree topologies. The evidence of character conflict among *Sasa veitchii, Acidosasa purpurea* and *Pleioblastus hindsii* sensu Nakai in the plastome Neighbor-Net analysis could be due to several possible reasons such as unstable conditions generated by heteroplasmy that arose through independent mutations in chloroplast genome or through biparental inheritance of plastomes or due to haplotype polymorphism (Wolfe and Randle, 2004). We also postulate that most of the character conflict among clades III, X and XII in the plastome dataset are due to the considerable amount of missing data (ca. 55.6%) in the *Indocalamus sinicus* (Clade X) plastome sequence. The exclusion of *Indocalamus sinicus* from the Neighbor-Net analysis revealed a much cleaner and consistent network (Appendix D: Supplementary Figure1).

The highly reticulate, narrowly-meshed network recovered for both *pvcel1* and *pabp1* could result from several causes. In a few recent studies (Triplett and Clark, 2010; Triplett and Clark, in revision), it was found that many species of the *Arundinaria*, *Shibataea* and *Phyllostachys* clades are hybrids of relatively recent origin and thus

hybridization plays a major role in understanding the relationships between these species and clades in Arundinarieae. However, there are no detailed studies of the other major clades, especially the old world temperate clades such as *Bergbambos*, the African alpine bamboos, *Thamnocalamus* and *Kuruna*. A thorough investigation of these clades with an emphasis on hybridization could provide more insight into their relationships.

Morphological character evolution

Based on our analysis, pachymorph rhizomes could consider as ancestral condition while the leptomorph rhizomes evolved multiple times within Arundinarieae and also in Bambusoideae. The pseudospikelets evolved independently at least twice within Arundinarieae. Despite the recognition of 12 lineages within the Arundinarieae, little work has been done on morphological evolution in the tribe due in large measure to the lack of resolution among these lineages. Rhizomes and pseudospikelets are two of the least well understood morphological traits in bamboos. Few rigorous discussions of rhizome structure are available (Rivière and Rivière, 1879; McClure, 1966; Stapleton, 1997); lack of study of rhizome structure is likely due to the fact that rhizomes are almost entirely or wholly underground. Similarly, detailed study of pseudospikelets has been limited in part due to the peculiar flowering cycles of woody bamboos. These two characters therefore rarely have been examined in an evolutionary context.

McClure (1966) used “pachymorph” and “leptomorph” terminologies to differentiate the 2 main rhizome systems in bamboos. Typically seen in clump-forming bamboos, pachymorph rhizomes have a defined neck (short or elongated) and a thick rhizome proper, which curves upward and terminates in an aerial culm. In contrast, the leptomorph rhizomes are characterized by long and slender axes with a single lateral bud

at every node, some of which grow into aerial culms. Stapleton (1997) considered only the leptomorph condition to represent “true” rhizomes, and he redefined pachymorph rhizomes as “pachymorph culm bases”. Early studies suggested that pachymorph rhizomes with cespitose tillering represented the ancestral condition (Holttum, 1956, 1958; Soderstrom, 1981; Wen, 1985). Current study provides evidence to support that the pachymorph rhizomes as the ancestral condition in Bambusoideae and Arundinarieae. It is evident that both Bambuseae and Olyreae are pachymorph while Arundinarieae include both pachymorph and leptomorph taxa (Fig. 4). Within Arundinarieae, there is a clear division between a group with mainly leptomorph rhizomes (Clades VI, VIII and IV) and a group with mainly pachymorph rhizomes (Clades V, III, XII, II, IX, X, I and VII), but at least one reversion to the other condition is seen in each of these groups. We also note that our sampling of the very diverse Clade V is very limited, which undoubtedly affects our inferences. Hence, we tentatively conclude that pachymorph rhizomes are the ancestral condition, in the evolutionary history of Arundinarieae and also in Bambusoideae.

In bamboos, the flower-bearing branches may have very complex structures. Of all the grasses, pseudospikelets, as typically defined, occur only in subfamily Bambusoideae. Conventional grass spikelets not only lack the branch buds at their base, but also remain as a single unit as they mature. In contrast each primary pseudospikelet usually develops into a tuft of pseudospikelets, with up to four or five orders of branching, as the basal buds start to produce higher order pseudospikelets. McClure (1966) defined a bamboo inflorescence as the “indeterminate” or “iteractant” type if they possess pseudospikelets and a bamboo inflorescence with regular “grass-like” spikelet as

the “determinate” or “semelauctant” type. Based on the mapping of reproductive structures on our plastome tree topology we were unable to draw a clear inference of the evolutionary history of pseudospikelets within Bambusoideae. Although it is still unclear whether the presence of pseudospikelets is ancestral or derived, we infer that pseudospikelets evolved independently at least twice in the evolutionary history of Arundinarieae. Even though the taxa of *Arundinaria* clade (Clade VI) that we sampled only consists of taxa those lack pseudospikelets, both presence and absence of pseudospikelets reported in Clade VI and also Clade V (*Phyllostachys*) (Clayton et al., 2006 onwards; Flora of China, 2006). Further analytical approaches are required to understand the origin of different components of floral morphology. However, there is a potential explanation for the presence of pseudospikelets in *Shibataea kumasaca* of the *Shibataea* clade (Clade IV). Triplett and Clark (in revision) speculated that the genus *Shibataea* may have an origin through hybridization, probably involving *Phyllostachys*, which was subsequently masked by introgression. Thus the presence of incomplete pseudospikelets in *Shibataea kumasaca* could have been inherited from its progenitors.

Conclusions

We investigated phylogenetic relationships within tribe Arundinarieae of Bambusoideae using both whole plastome sequence data and low-copy nuclear markers. Both data sets supported most of the previously recognized major clades with few exceptions. Except for Clades IV and V, the low-copy nuclear gene phylogeny resolved Clades I, II, III, VI, VII and XII, although relationships among these clades differed in some respects from the plastome phylogeny. These conflicting signals, as tested by taxon removal experiments, alternative hypothesis testing and Neighbor-Net analyses, reflect

the complex relationships among these taxa; probably a combination of factors, mainly recent hybridization and incomplete lineage sorting, has produced this complexity. Further, based on the analyses of morphological character evolution, we propose that pachymorph rhizomes as the ancestral condition and the pseudospikelets evolved independently a minimum of two times during the evolutionary history of Arundinarieae.

Due to some of the inconsistent and complex results obtained for the low-copy nuclear data, we suggest that further studies needed to understand especially how hybridization events impacted the entire Arundinarieae tribe. In addition, future studies of morphological evolution are needed but these will require better sampling within Clade V.

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Zhu. Q., Ge, S., 2005. Phylogenetic relationships among A-genome species of the genus *Oryza* revealed by intron sequences of four nuclear genes. New Phytologist 167, 249–265. http://dx.doi.org/10.1111/j.1469-8137.2005.01406.x.

APPENDIX A. SPECIMEN VOUCHER INFORMATION AND GENBANK ACCESSION NUMBERS OF PLASTOME SEQUENCES

Shaded boxes represent the GenBank sequence submissions in progress.

Species	Lineage	Genome size bp)	GenBank Accession number	Voucher
Acidosasa purpurea	VI	139,697	HQ3377	N/A
Ampelocalamus calcareus	XI	139,689	KJ496369	N/A
Arundinaria appalachiana	VI	139,547	KC817462	N/A
Arundinaria gigantea	VI	138,935	JX235347	N/A
Arundinaria tecta	VI	139499	KC817463	N/A
Bambusa bambos	Bambusinae	139,606	KJ87098	N/A
Bergbambos tessellata	I	139,441		LC1319
Chimonocalamus sp.	III	139,682		Clark & Keiners s.n.
Chusquea spectabilis	Chusqueinae	136,848	KJ870990	N/A
Cryptochloa strictiflora	Olyrinae	135,033		N/A
Fargesia nitida	VI	139,559		J. M. Saarela 591531
Ferrocalamus rimosivaginus	IV	139,467	HQ3377	N/A
Gaoligongshania megalothyrsa	IX	140,064	JX513419	N/A
Hordeum vulgare	Pooideae	136,462	NC008590	N/A
Indocalamus sinicus	XI	117,890	KJ531442	N/A
Indocalamus wilsonii	VIII	139,962	JX513421	N/A
Kuruna debilis	XII	139,694		LA123-5
Kuruna densifolia	XII	139,652		LA 130-3
Lolium perenne	Pooideae	135,282	NC009950	N/A
Melocanna baccifera	Melocanninae	139,619		XL&LC930

APPENDIX A continued.

Species	Lineage	Genome size bp)	GenBank Accession number	Voucher
Oldeania alpina	II	139,602		LA170
Olyra latifolia	Olyrinae	136,785	NC024165	N/A
Phyllostachys aurea	V	139,695		LA172
Pleioblastus hindsii sensu Nakai	VI	139,682		LC1317
Sasa veitchii	VI	139,731		LC1325
Shibataea kumasaca	IV	139,717		LC1290
Thamnocalamus spathiflorus	VIII	139,498	KJ871005	N/A

APPENDIX B. SPECIMEN VOUCHER INFORMATION AND GENBANK ACCESSION NUMBERS OF LOW-COPY NUCLEAR GENES

Missing data are indicated by dashes and newly published sequences are highlighted in **bold**. Underlined GenBank accession numbers are the H homeologs. Shaded boxes represent the GenBank sequence submissions in progress.

Specimen name	Voucher	GenBank Accession numbers										
		pabp1				*pvcel1*				*rpb2*		
		C copy	D copy	A copy	B copy	C copy	D copy	A copy	B copy	C copy	D copy	A copy
Arundinaria gigantea	JT 197	*	*	KM209011	KM208993	*	*	KM209119	KM209088	*	*	-
Arundinaria tecta	JT 24	*	*	KM209012	KM208992	*	*	KM209120	KM209083	*	*	-
Bergbambos tessellata	JT 202	*	*	KM209002	KM208988	*	*	KM209104	KM209070	*	*	-
Brachyelytrum erectum	JT 199	KM208956	*	*	*	KM209031	*	*	*	H copy	*	*
*Chimonocalamus delicates*_1	JT 275	*	*		-	*	*	-	-	*	*	
*Chimonocalamus delicates*_2	JT 275	*	*		-	*	*			*	*	-
Chimonocalamus montanus	JT 261	*	*	KM209004	KM208990	*	*	KM209103	KM209072	*	*	-
Chimonocalamus pallens	9206	*	*			*	*			*	*	
Chusquea bambusoides	LC1029	KM208987	-	*	*	-	-	*	*	-	-	*
Chusquea scandens	LC & XL 1235	-	-	*	*	KM209069	KM209052	*	*	-	-	*
Chusquea spectabilis	LC919	-	KM208973	*	*	KM209065	KM209049	*	*		-	*
Fargesia nitida	JT 222	*	*			*	*			*	*	
Ferrocalamus rimosivaginus	JT 277	*	*			*	*		-	*	*	
Guadua angustifolia	LC & XL 931	KM208983	KM208970	*	*	KM208983	KM208970	*	*	-	-	*
Kuruna densifolia	LA 126	*	*	KM209003	KM208989	*	*	KM209102	KM209071	*	*	-

APPENDIX B continued.

Specimen name	Voucher	GenBank Accession numbers										
		pabp1				***pvcel1***				***rpb2***		
		C copy	**D copy**	**A copy**	**B copy**	**C copy**	**D copy**	**A copy**	**B copy**	**C copy**	**D copy**	**A copy**
Kuruna floribunda	LA 135-5	*	*			*	*			*	*	
Oldeania alpina	Faden et al.	*	*			*	*			*	*	
*Olyra latifolia*_1	XL & LC 911	KM208958	*	*	*	KM208958	*	*	*	*	*	*
*Olyra latifolia*_2	XL & LC 911	KM208959	*	*	*	KM208959	*	*	*	*	*	*
*Phyllostachys bambusoides*_1	JT 121	*	*	KM209007	-	*	*	KM209111	KM209080	*	*	-
*Phyllostachys bambusoides*_2	JT 121	*	*	KM209009	-	*	*	KM209116	KM209077	*	*	-
Pleioblastus chino	JT 420	*	*	KM209013	KM208999	*	*	KM209125	KM209092	*	*	-
Pleioblastus gozadakensis	JT 344	*	*	KM209014	KM208997	*	*	KM209013	KM209097	*	*	-
Pleioblastus hindsii_1	JT 411	*	*	KM209015	-	*	*	-	KM209099	*	*	-
Pleioblastus hindsii_2	JT 411	*	*	KM209016	KM208998	*	*	KM209124	-	*	*	-
*Pleioblastus maculatus*_1	JT 252	*	*	KM209021	KM209000	*	*	KM209121	KM209089	*	*	-
*Pleioblastus maculatus*_2	JT 252	*	*	-	-	*	*	-	KM209090	*	*	-
Pleioblastus simonii _1	JT 410	*	*		-	*	*	-		*	*	
*Pleioblastus simonii*_2	JT 410	*	*		-	*	*		-	*	*	-

APPENDIX B continued.

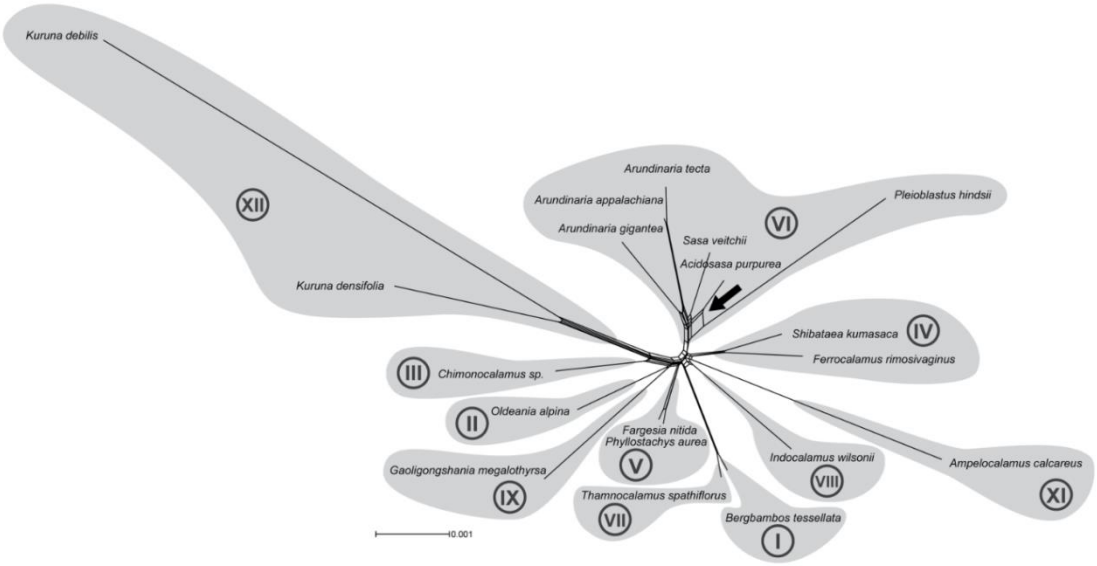
Specimen name	Voucher	GenBank Accession numbers										
		pabp1				***pvcel1***				***rpb2***		
		C copy	**D copy**	**A copy**	**B copy**	**C copy**	**D copy**	**A copy**	**B copy**	**C copy**	**D copy**	**A copy**
*Pseudosasa amabilis*_1	JT 545	*	*	KM209022	-	*	*	KM209122	KM209091	*	*	-
*Pseudosasa amabilis*_2	JT 545	*	*	KM209023	KM209001	*	*	-	-	*	*	-
*Pseudosasa japonica*_1	JT 320	*	*	KM209017	KM208994	*	*	KM209127	KM209101	*	*	-
*Pseudosasa japonica*_2	JT 320	*	*	KM209029	-	*	*	KM209106	KM209076	*	*	-
*Sasa longiligulata*_1	Zeng 06197	*	*		-	*	*	-	-	*	*	
*Sasa longiligulata*_2	Zeng 06197	*	*			*	*			*	*	-
*Sasa veitchii*_1	JT 126	*	*	KM209024	-	*	*	-	-	*	*	-
*Sasa veitchii*_2	JT 126	*	*	KM209025	KM208991	*	*	KM209107	KM209084	*	*	-
Sasamorpha borealis	JT 294	*	*	KM209030	KM208995	*	*	KM209105	KM209075	*	*	-
*Shibataea chinensis*_1	JT 231	*	*	KM209006	-	*	*	KM209114	KM209073	*	*	
*Shibataea chinensis*_2	JT 231	*	*	KM209005	-	*	*	KM209115	KM209074	*	*	-
*Shibataea kumasaca*_1	JT 230	*	*		-	*	*	-	-	*	*	
*Shibataea kumasaca*_2	JT 230	*	*		-	*	*	-	-	*	*	-
Thamnocalamus spathiflorus	LC3119	*	*			*	*			*	*	
*Yushania ambositrensis*_1	1335	*	*			*	*		-	*	*	
*Yushania ambositrensis*_2	1335	*	*	-	-	*	*		-	*	*	-
Yushania niitakayamensis	RM 25	*	*			*	*		-	*	*	

APPENDIX C. ANALYSIS OF MORPHOLOGICAL EVOLUTION DATA MATRIX

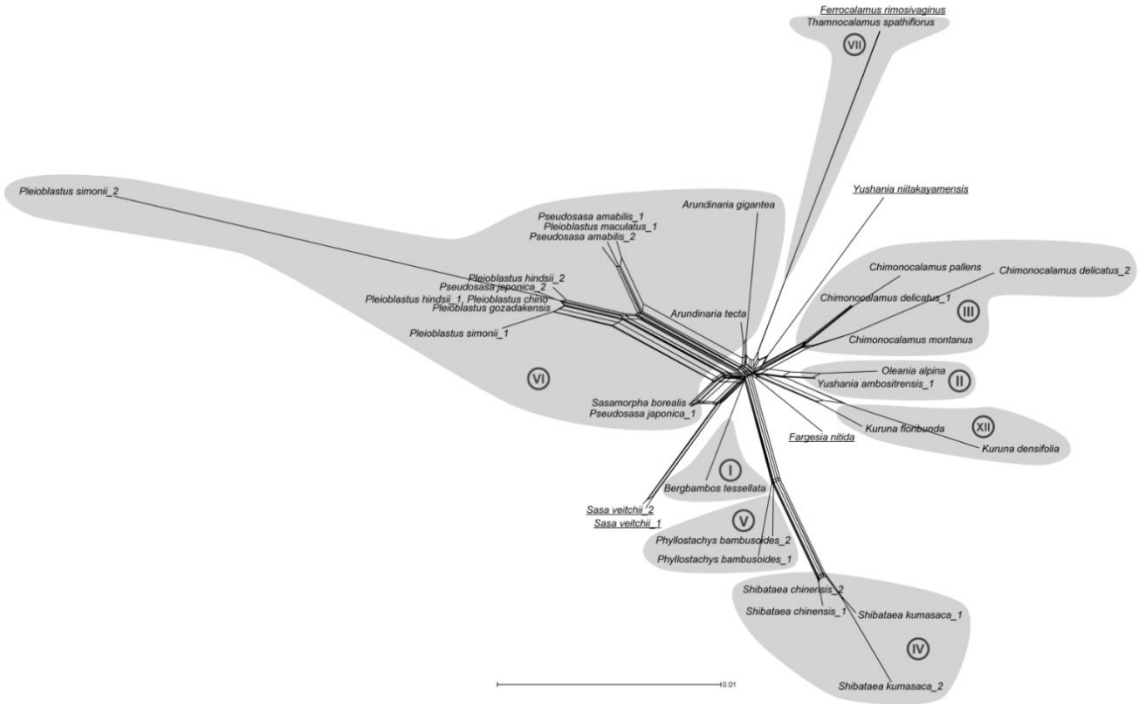
0 and 1 in "Rhizomes" column represents presence of pachymorph and leptomorph rhizomes respectively. Absence of the pseudospikelets, incomplete pseudospikelets and complete pseudospikelets are scored as 0,1 and 2 respectively in "Pseudospikelets" column. Outgroups are shaded in gray. "-" represents "not applicable". ? indicates unknown character states.

Species	Rhizomes	Pseudospikelets
Acidosasa purpurea	0	0
Ampelocalamus calcareus	1	?
Arundinaria appalachiana	0	0
Arundinaria gigantea	0	0
Arundinaria tecta	0	0
Bambusa bambos	1	2
Bergbambos tessellata	1	0
Chimonocalamus sp	1	0
Chusquea spectabilis	1	0
Cryptochloa strictiflora	1	0
Fargesia nitida	1	0
Ferrocalamus rimosivaginus	0	0
Gaoligongshania megalothyrsa	1	0
Hordeum vulgare	-	0
Indocalamus wilsoni	0	0
Indocalamus sinicus	0	0
Kuruna debilis	1	0
Kuruna densifolia	1	0
Lolium perenne	0	0
Melocanna baccifera	1	2
Oldeania alpina	1	0
Olmeca reflexa	1	0
Olyra latifolia	1	0
Phyllostachys aurea	0	2
Pleioblastus hindsii sensu Nakai	0	0
Sasa veitchii	0	0
Shibataea kumasaca	0	1
Thamnocalamus spathiflorus	1	0

APPENDIX D. SUPPLEMENTARY FIGURES



Supplementary Figure 1. Neighbor-Net analyses of Arundinarieae based on plastome data with the exclusion of *Indocalamus sinicus*. Roman numerals represent the major Arundinarieae clades. Arrow indicates the character conflicts.



Supplementary Figure 2. Neighbor-Net analyses of Arundinarieae based on *pvcel1* low-copy nuclear gene data with exclusion of the two alleles of *Sasa longiligulata*. Roman numerals represent the major Arundinarieae clades.The underlined taxa are the ones that are placed out of their corresponding clades showing disagreements.

CHAPTER 5

SIMPLE WEB-BASED INTERACTIVE KEY DEVELOPMENT SOFTWARE (WEBIKEY) AND AN EXAMPLE KEY FOR *KURUNA* (POACEAE: BAMBUSOIDEAE)[1]

A manuscript submitted to *Applications in Plant Sciences*

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Abstract

- *Premise of the study:* Programs that are user-friendly and freely available for developing web-based interactive keys are scarce and most of the well-structured applications are relatively expensive. WEBiKEY was developed to enable researchers to easily develop their own web-based interactive keys with fewer resources.
- *Methods and Results:* A web-based multi-access identification tool (WEBiKEY) was developed that uses freely available, Microsoft ASP.NET technologies and an SQL Server database. WEBiKEY was tested for its usability with a sample dataset, the temperate woody bamboo genus *Kuruna*.
- *Conclusions:* WEBiKEY is freely available to the public and can be used to develop web-based interactive keys for any group of species. The interactive key we developed for *Kuruna* using WEBiKEY enables users to visually inspect characteristics of *Kuruna* and identify an unknown specimen as one of seven possible species in the genus.

Keywords: Bamboos, identification tools, interactive keys

Introduction

Plant and animal identification keys are used by many biologists to assign a scientific name to a biological specimen (Dallwitz, 1980; Jarvie and Stevens, 1998; Dallwitz et al., 2000 –onwards; Heidorn, 2001). The most common form of identification key used in printed publications is the dichotomous key, which presents a series of choices or couplets that must be followed in order (*e.g.*, the dichotomous key for *Kuruna* in Attigala et al., in press). In contrast, an interactive key supports multiple access points where users are allowed to choose the characters in any order and only use the characters available on the specimen. DELTA-format (Description Language for Taxonomy: Dallwitz et al., 1993 –onwards) interactive keys are available for many species (http://www.delta-intkey.com/); however, these require installation of the DELTA INTKEY application on the user's personal computer (PC). There are commercially available interactive key development programs available, such as LucID (http://www.lucidcentral.com/en-us/home.aspx) and IdentifyIt (http://www.eti.uva.nl) but they are relatively expensive (ca. $600 USD). While programs like VisualKey (http://insects.ummz.lsa.umich.edu/beemites/vk_bees/vk_bees.htm), MEKA(http://ucjeps.berkeley.edu/meacham/meka/) and Navikey (http://www.navikey.net/) are freely available, these often require helper applications, plug-ins, Java applets, and web forms, resulting in lengthy download times from the server to the client computer. In addition, some of these free interactive key programs are

complex to run and difficult to use. We chose *Kuruna* to trial our identification key because it is a small group of species that often need to be identified by Sri Lankan biologists, there is no interactive key currently available for the genus, and identifying bamboos requires the use of many unique characters that may not be familiar to biologists. Also, currently, there is a web-based multi-access identification key for the flowering plants of Sri Lanka that includes 438 species and uses a different interactive key format (KeyBase: http://keybase.rbg.vic.gov.au/key/project/8). However, the KeyBase key excludes the *Kuruna* species found in Sri Lanka. There are seven species in *Kuruna* (Attigala et al., 2014; Attigala, et al., in press) found in Sri Lanka and south India.

We designed a web-based multi-access identification tool (WEBiKEY) that uses Microsoft ASP.NET technologies and an SQL Server database, both of which are available online as free downloads. The information related to the study group can be imported to WEBiKEY as spreadsheets (.xlsx). WEBiKEY was tested for its usability with a sample dataset, the temperate woody bamboo genus *Kuruna*. This simple, easy-to-use interactive key enables users with plant material from an unknown species in *Kuruna* to visually inspect characteristics of the bamboo and identify it as one of seven possible species in the genus. The images used in the *Kuruna* interactive key ensure that the characters and unique character states are easy to understand.

Methods and Results

Development of the web-based multi-access interactive identification key (WEBiKEY)

Our current approach uses Microsoft ASP.NET technologies and an SQL Server database that are freely available online. ASP.NET contains Microsoft's latest framework and tool sets for building web-based applications. SQL Server is a robust, fast, and secure relational database, which is also available as a free download in its Express Edition. We developed a set of entity relations and an entity relation diagram (ERD) for the database (Fig. 1). The database consists of four tables: *CharacterCategory*, *Character*, *CharacterState,* and *Species*. The characters are classified into a few major groups based on vegetative and reproductive morphology. Each group is then represented by a row in the *CharacterCategory* table. The *Character*, *CharacterState,* and *Species* tables contain the names of the characters, the names of the variable character states, and the names of the species. Since a character category can contain one or more characters, the relationship between these tables have a "one-to-many" relationship. However, the relationship between the *CharacterState* and *Species* tables is "many-to-many" because a species can have many character states and a character state can be shared by many species. The WEBiKEY application is intended for two types of users: end-users and users with administrative privileges (Admins). Admins can set-up the application by uploading all of the species information and character state details with images. The characters and character states can be imported as spreadsheets (.xlsx). Setting up the application for the Admin's desired plant or animal group is explained in the "Readme" documentation (*see Appendix*) in the installation package. The Admin is also responsible

for hosting the developed application on a server, where end-users can access it via the Internet to identify their unknown species. This program is capable of handling large amounts of species and character data. Even though there are no theoretical limitations to the number of species in a key, there may be practical limitations due to the hosting system's resources (hard disk space, memory, processing power, etc.). The ERD (Fig. 1) illustrates the database structure and its tables with relationships that ensure data integrity and handle dynamic data changes such as insertions, deletions, and updates.

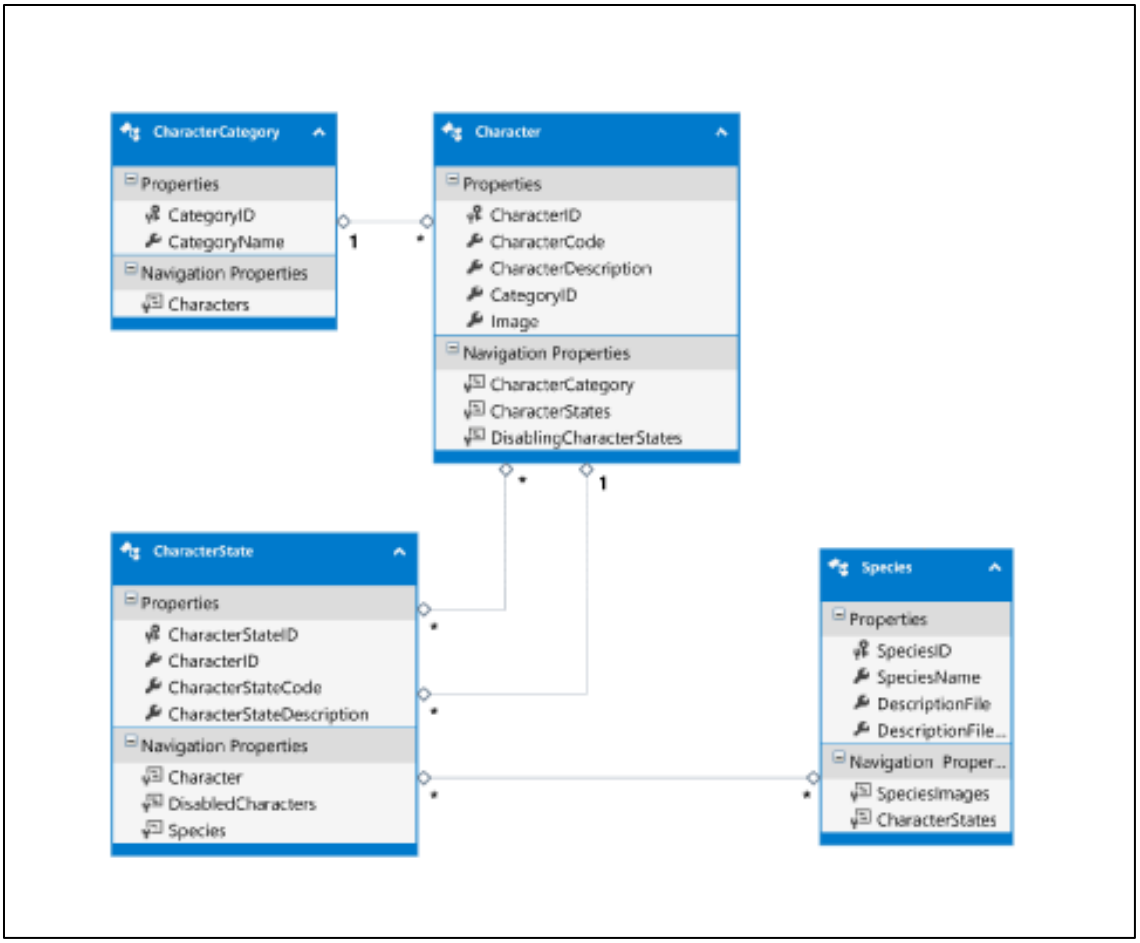

Fig. 1. Entity relation diagram (ERD) of the database design. Rectangles represent Entities with their attributes and the lines connecting these rectangles represent relationships among these Entities. The numbers 0,1 and the "*" indicate the relationship type. For example, the relationship between *CharacterCatergory* and *Character* is a one-to-many, i.e., one character category can have multiple characters, while each character must have only one character category.

Testing the WEBiKEY key for the bamboo genus *Kuruna*

The WEBiKEY key for the bamboo genus *Kuruna* has three main web pages: a home page (Fig. 2A), a major character group selection page (Fig. 2B) and a detailed interactive key page (Fig. 2C), along with three menus that allow users to download each *Kuruna* species description (Fig. 2D), a dichotomous key (Fig. 3A) or a glossary in PDF form (Fig. 3B). There is also a "Help" link that provides details about how to use the interactive key. The home page of the *Kuruna* key gives a brief description about bamboos in general and about the genus *Kuruna*. The choice of morphological characters for this study was based on the characters used in the Bamboo Biodiversity website [Bamboo Phylogeny Group (BPG), 2005]. Bamboos have unique characters that require special botanical terms. Thus, illustrations and images are significant components of this interactive key and were reproduced from the Bamboo Biodiversity website (BPG, 2005) with permission. Fifty five different characters were used in an interactive key to distinguish the seven *Kuruna* species in Sri Lanka and south India.

The end-user can first click on the "Interactive key" link from the menu which directs the user to a web page that lists the eight major types of characters for temperate woody bamboos (Fig. 2B). The user must select which major character groups are present in the specimen that needs to be identified. The program will use this selection to display only the detailed characters and their character states for each major group (*e.g.*, if the specimen does not have flowers, only the vegetative characters will be displayed). This page also contains an abstract drawing of a typical bamboo plant with the major characters labeled. By clicking the next button on this page, the user will be directed to the third page (Fig. 2C). This serves as the detailed interactive key for *Kuruna*. The

characters and character states corresponding to the major character groups that the user selected are in the top left panel, while images of the character states are displayed in the top right panel. The bottom left and right panels display in real-time the matching and eliminated species based on the user's selection of character states. Another "intelligent" feature of the interactive key is that if the user selects a character state that determines the availability of another character, the user may or may not be able to select the dependent character states depending on the first character state selection. For example, if the user selects subtending bracts at the base of axis bearing the spikelet: absent, then they would not be able to select the other character states related to subtending bracts, such as subtending bract morphology (Fig. 2C). This allows the users to make fewer mistakes and makes the key easier to use. Furthermore, comparing the character states with the images further enhances the user-friendliness of this application. Under "View Species Info" menu item, users can download descriptions of species which have recently been published in Attigala et al. (in press), as well as images and illustrations, if they are available (Fig. 2D). Users may also click "View Dichotomous Key" to see a traditional dichotomous key for the genus. By clicking "Glossary", users can see written descriptions for the scientific terminology used in the interactive key of *Kuruna*. This WEBiKEY interactive key of *Kuruna* is available at http://webikey.agron.iastate.edu/.

Minimum system requirements, known issues and workarounds

Readers can download the program source code and its database at https://github.com/WEBiKEY/InteractiveKey and use it to develop their own web-based interactive keys. The minimum requirements for end-users who want to use the WEBiKEY of *Kuruna* are access to a computer with any operating system, a web

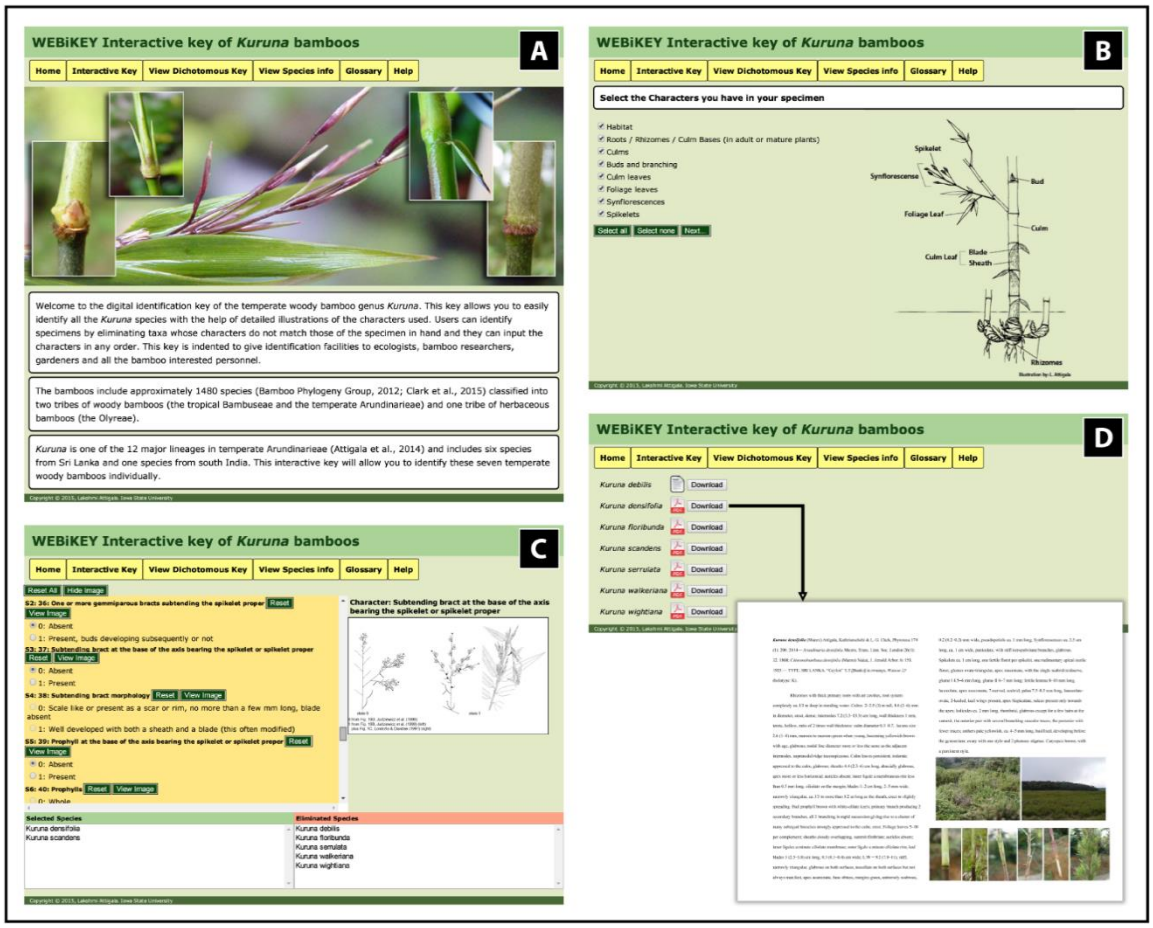

Fig. 2. Online pages of the multi-access web-based interactive identification key (WEBiKEY) of *Kuruna*. A- Home page; B- Major character group selection page; C- Detailed web based interactive key of *Kuruna*; D- "View Species info" page with a PDF opened for *Kuruna densifolia*.

browser and an internet connection. Developers and hosts of an interactive key need to have the following system requirements: a system running Windows 7 or a newer operating system (OS), .NET Framework 4.5 or a newer version (freely available at: http://www.microsoft.com/en-us/download/details.aspx?id=42642), IIS Express 7 or a newer version (Component of Windows OS), SQL Server Express 2008 R2 or a newer version (freely available at: http://www.microsoft.com/en-us/server-cloud/Products/sql-server-editions/sql-server-express.aspx), Visual Studio Express for Web 2013 or a newer version is recommended if users are planning to edit the source code (Freely available at: https://www.visualstudio.com/en-us/products/visual-studio-express-vs.aspx) and

Microsoft Excel 2010 or a newer version is required to create and edit worksheets that are uploaded to the application, such as: characters, character states and species information. However, there is only one known issue with the interactive keys developed using WEBiKEY. It is always required to click on the radio button itself to select character states instead of clicking on the text next to the radio button. Clicking on the text causes the program to change the first character state selection. This is an issue with the way ASP.NET Web Forms renders radio button groups and might be addressed in future ASP.NET updates. The authors will update the downloadable source code when they find a solution. Further, currently WEBiKEY is only developed for the Windows platform. The current program was compared with some freely available interactive key development applications (Intkey, MEKA and NaviKey) (Table 1). Some comparison features listed in Table 1 were adopted from Dallwitz (2000 –onwards) along with additional features.

Conclusion

The simple web-based multi-access interactive identification software we developed is freely available to the public and can be used to develop web-based interactive keys for any group of species. Compared to other free interactive key development software WEBiKEY is easier to use for the Admin and User. Some of the important user-friendly features of the software are the ability to upload spreadsheets to the database, character state illustrations can be displayed, illustrations of any size can be scaled, a glossary available to help with unfamiliar terms, character dependencies were addressed and an extensive amount of text can be incorporated to aid interpretation of the characters.

TABLE 1. A comparison between WEBiKEY and a few other freely available interactive key development programs. **Bold** features denote essential features for identification. ** and * denote very important and important features for identification respectively. Features not in boldface nor with *s are useful for identification, but not necessary.

Features	WEBiKEY	Intkey	MEKA	NaviKey
Retaining taxa for which a character is not recorded when that character is used	√	√	√	√
No restrictions on the order of the character use**	√	√	√	√
Removing or changing characters used *	√	√	√	√
Character dependencies*	√	√	-	√
Detailed explanation to aid interpretation of characters conveniently available within the system*	√	√	-	-
Glossaries available*	√	-	-	-
Displaying illustrations of character states*	√	√	-	-
Illustrations of any size can be scaled*	√	√	-	-
Directly importing datasets via spreadsheets (.xlsx)*	√	-	?	-
Numbers of taxa, characters, and states are unlimited*	√	√	?	?
The lengths of fields (e.g., taxon names, text of characters, character notes) are unlimited*	√	√	-	√
Fast execution*	√	√	√	-
Running without illustrations (a package normally containing illustrations can run well without them)	√	√	-	-
Text files attached to taxa	√	√	-	-
Complete, built-in help available	√	√	-	√
No special memory requirements	√	√	√	√
Mainly intended as a web-based application	√	√	-	√
Characters are categorized into major groups, allowing users to select only the major parts in an unknown (e.g., flowers, fruits, etc.)	√	-	-	-
Users can access conventional dichotomous keys	√	-	-	-

Note: √= feature is implemented in the program; -= feature in not implemented in the program; ? = feature unknown.

In addition, there are a few other features that are not necessary, but useful for the performance of the program such as the characters are categorized in to major groups,

users can access conventional dichotomous keys, text files can be attached to taxa and the program can run without illustrations. Our WEBiKEY application was successfully used to create a web-based interactive key for all the species of the bamboo genus *Kuruna* to test its effectiveness. Despite the availability of KeyBase, none of the Sri Lankan high schools or universities use any form of electronic identification keys frequently to identify flora or fauna. However, some universities and very few high schools teach about interactive keys (pers. comm.). In addition, there are only two studies that discuss native Sri Lankan bamboos (Soderstrom and Ellis, 1988; Attigala et al., 2014) and there is only one pictorial guide to identify some of the economically important bamboos in Sri Lanka (De Zoysa and Vivekanandan, 1994). Thus, we believe that providing an online key to *Kuruna* will be important for scientists, students, gardeners, conservationists, etc. in Sri Lanka. The interactive nature of the *Kuruna* key, the ability to easily view images of the characters, and the large amount of information about each species makes it easier to teach students about bamboo morphology.

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APPENDIX. "README" DOCUMENTATION OF WEBIKEY

WEBiKEY (R) version 1.0 (11/09/2015)

GENERAL USAGE NOTES

WEBiKEY is a web application that can be used to develop simple web based multi-access interactive identification tool for any taxonomic group.
This document describes how to setup your own hosting environment using IIS so that WEBiKEY can be hosted on your local computer. If you wish to use a 3rd party hosting service, skip steps 2 and 3.

MINIMUM SYSTEM REQUIREMENTS FOR THE HOSTING ENVIRONMENT

- A system running Microsoft Windows 7 or a newer operating system.
- .NET Framework 4.5 or a newer version (Freely available at: http://www.microsoft.com/en-us/download/details.aspx?id=42642).
- IIS 7 or a newer version (Component of Windows Operating System)
- SQL Server Express 2008 R2 or a newer version (Freely available at: http://www.microsoft.com/en-us/server-cloud/Products/sql-server-editions/sql-server-express.aspx)
- Visual Studio Express for Web 2013 or a newer version is recommended if users are planning to edit the source code (Freely available to download at: https://www.visualstudio.com/en-us/products/visual-studio-express-vs.aspx)
- Microsoft Excel 2010 or a newer version is required to create and edit worksheets containing the information that are uploaded to the application, such as: characters, character states and species information.

INSTALLATION INSTRUCTIONS

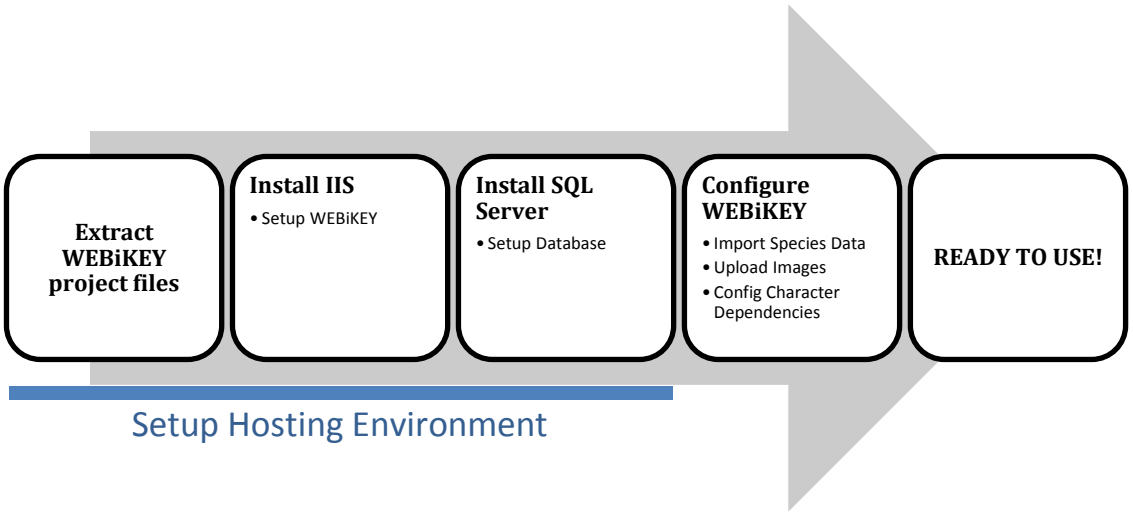

Figure 1- Summary of the installation and setting up WEBiKEY program

You must have an account with administrative privileges to complete these installations.

1. **Extract the WEBiKEY project files to a suitable folder.**
2. **Install IIS Server**

Make sure you have IIS installed on your computer. If not, go to Turn Windows features on or off, check Internet Information Services and, under Web Management Tools, make sure IIS Management Console is also checked.

3. **Setup website**

Open IIS Manager from your Start menu. Expand the computer name root item under connections panel. For security and isolation purposes, it is recommended to create a dedicated Application Pool for your website. For that, right click on Application Pools node and click Add Application Pool…. On the Add Application Pool dialog, provide a name for your Application pool and select .NET CLR Version 4 for .NET CLR Version. For Managed pipeline mode, select Integrated and click OK to complete the dialog.

Right click on the Default Web site under Sites node and click Add application… to add a new website to your IIS Server. Provide an Alias for your site and make sure to select the application pool you created in the previous step. For the physical path, browse and select the "WebSite" sub-folder under the WEBiKEY project folder. Click OK to complete the dialog.

4. **Install SQL Server Express Edition**

Download and install the free version of Microsoft SQL Server (Express Edition). When downloading, select the SQL Server Express with Tools option. Run the setup and complete the installation.

Open SQL Server Management Studio from the Start menu and connect to your local database server instance. In Object Explorer, right click on Databases and select Attach…. Click Add... to browse and select the WEBiKEY.mdf file in Database sub-folder under the WEBiKEY project folder. Click OK to finish attaching the database. Now, from your browser, navigate to http://localhost/[Alias you provided in the previous step] to view the website. However, until you import your data on to the website, the interactive key is not functional.

Using a 3rd party hosting provider

If you decide to use a 3dr party hosting provider, make sure your hosting environment is capable of hosting ASP.NET 4.5 applications and SQL Server 2008 R2 or higher databases.

Getting started

If you have followed the previous steps, or have your own hosting provider, now you should be able to view the website by going to the appropriate URL. In the case of hosting on your local computer, as described earlier, navigate to http://localhost/*[Alias]* to view the website.

When specifying URL's, this document assumes that you have hosted WEBiKEY locally. In the case of a 3rd party hosting provider, please replace "localhost" with your domain name in all URL's as specified by your hosting provider.

Admin Login

Admin section of the website provides the ability to upload and administer data and files on the website. To access the admin side, navigate to http://localhost/*[Alias]/admin/*.

Enter administrator username and password to login. The default administrator login is: Username: admin, Password: Password!2.

Import data from Microsoft Excel file

All information on the website can be imported from Excel files. There are two data import pages on the website: Import Characters / States and Import Species / States. This website only supports Microsoft Excel 2007 or newer files and the first worksheet must contain data. If the workbook has more than one worksheet, only the first sheet will be used by the system to import data.

Import process is designed as a wizard that will guide you through the import process. On the first step, click Browse… and select the Excel file that contains data. If you are adding more data to the database and none of the information exists already in the database, select Append. Otherwise select Overwrite Existing Data. Click Next.

The second step of the wizard is to map columns between the Excel sheet and the database. This gives you the flexibility of having anything as column headers (except for importing the species and their character states, which requires specific column names as described below in step 2). The first column shows you the list of column headers found in the Excel worksheet. The second column contains list of database items that the Excel column can be mapped to. If you want to ignore information in a particular column in the Excel, select --ignore-- as the database column from the list. In the following example, Notes column in the Excel worksheet is ignored.

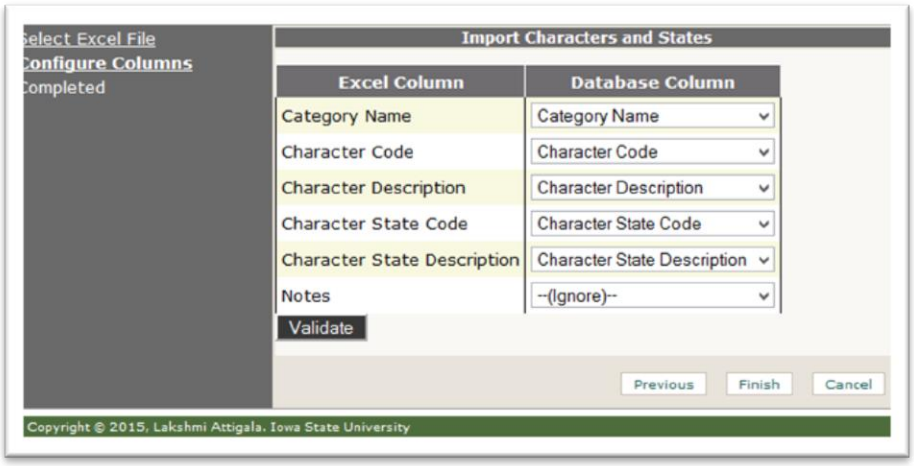

Figure 2 - Page that allows Admin to map columns between the Excel sheet and the database. Click Finish to begin importing data.

1. Import Characters, Character States and Character Categories

Table 1- A sample data table that can be imported into the website using Excel.

Character Code	Character Description	Characte State Code	Character State Description
H1	Habitat:	0	Understory
H1	Habitat:	1	Open grassland
H1	Habitat:	2	Open rocky plains
R1	Primary root air canals:	0	Absent
R1	Primary root air canals:	1	Present
R2	Pachymorph rhizomes	0	Absent
R2	Pachymorph rhizomes	1	Present
R3	Culm base morphology	0	Slender (all internodes more or less equal in diameter) and more or less vertical
R3	Culm base morphology	1	At least some proximal internodes thicker than the distal internode(s) emerging from the soil and more or less horizontal (pachymorph)
R4	Culm base branching (tillering):	0	Tillering absent
R4	Culm base branching (tillering):	1	1 tiller per culm base present
R4	Culm base branching (tillering):	2	2 or more tillers per culm base present
R5	Culm neck development:	0	Short (neck < the length of the culm base section with relatively short, bud-bearing internodes)
R5	Culm neck development:	1	At least some culm necks long (neck > the length of the culm base section with relatively short, bud-bearing internodes)
C1	Habit:	0	Erect
C1	Habit:	1	Apically arching/pendulous
C1	Habit:	2	Clambering/scandent
C1	Habit:	3	Twining
C1	Habit:	4	Decumbent
C2	Culm internodes:	0	All solid (at least when young)
C2	Culm internodes:	1	All hollow
C2	Culm internodes:	2	Some proximal internodes (including the basalmost ones) solid, distal internodes hollow

2. Import Species and their Character States

Create a column for each character and use character code as the column header. For each species and each character code, put the character state code in the corresponding cell.

Table 2- A sample data table which can be used as a template to import species and their character states to the system.

	H1	**R1**	**R2**	**R3**	**R4**	**R5**	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
Kuruna debilis	0	0	1	1	2	0	2	1	3	0	0	1	1
Kuruna densifolia	1	1	1	1	2	0	0	1	3	0	0	0	0
Kuruna floribunda	0	0	1	1	2	0	0	1	2	0	0	1	1
Kuruna scandens	0	0	1	1	2	0	2	1	4	0	0	1	0
Kuruna serrulata	2	0	1	1	2	0	0	2	2	0	0	1	0
Kuruna walkeriana	0	0	1	1	2	0	0	1	3	0	0	1	0
Kuruna wightiana	0	0	1	1	2	0	0	1	2	0	0	1	1

Note. For the species import process to work properly, you must import all the characters and character states prior. Leave the corresponding cell on the worksheet blank for unknown or not applicable data.

Uploading character images

This website allows you to upload an image for each Character in the database.

To upload character images, in the Admin section, click Upload Character Images…. First select the character code that you want to upload an image for from the dropdown list. If that character already has an image in the database, it will be shown below. If you decided not to have an image for the selected character, click Remove Image. If you want to upload a new image for the character, click Browse…, select the image and click Upload.

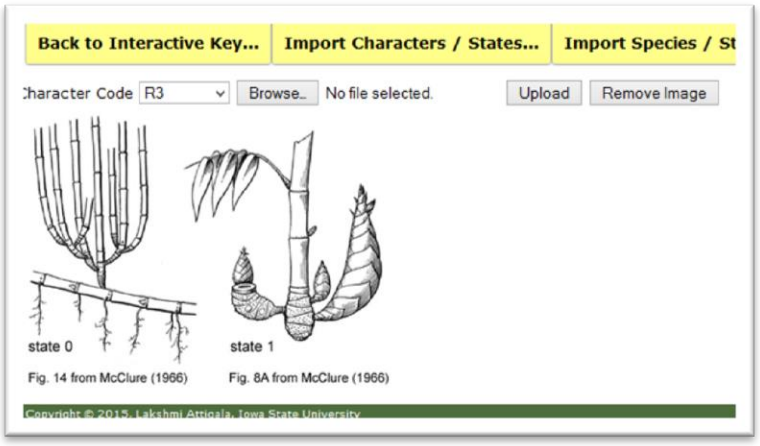

Figure 3 - Page that allows Admin to upload image for each Character in the database

Configure Character Dependencies

Suppose character B is not applicable when the character state 2 is selected for character A, then the website allows you to configure these types of character dependencies through the Admin pages. Click Configure Character Dependencies… to go to character dependency configuration page.

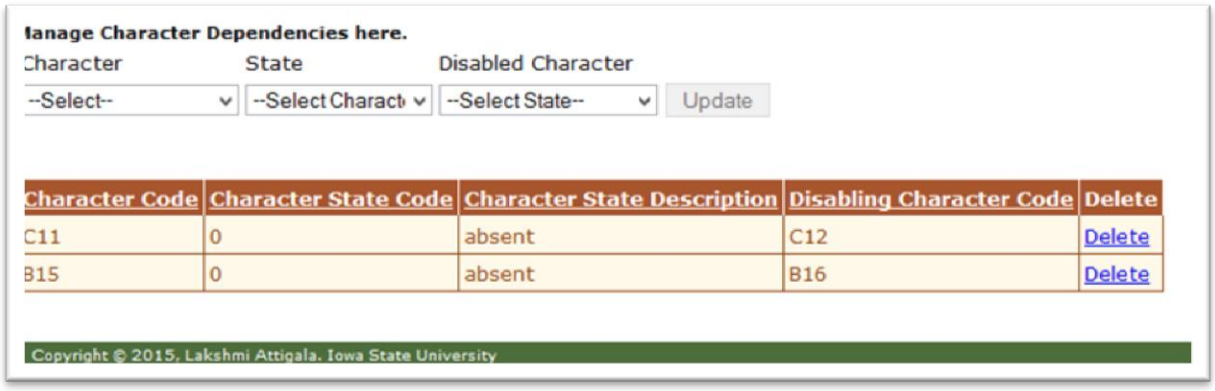

Figure 4 - Page that allows Admin to configure the character dependencies.

First, select a character and one of its character states from the first two dropdown lists. From the third dropdown, select the character that you want to be not applicable when this character state is selected. Then click update to add this dependency to the system.

The list of dependencies that are already in the system is also shown below. Click the Delete link, if you want to remove that character dependency from the system. According to our example scenario, select “Character A” from the first dropdown and then select “2” from the next list. Select “Character B” as the Disabled Character from the third dropdown. Once this configuration is added to the system, when a user select the character state 2 for Character A, they will not be able to select any character states for Character B, and it will be grayed out.

Editing the Home page contents

You can use your favorite HTML editor to change the contents of the home page. Simply open the Default.aspx in the root folder of the extracted web project files and make desired changes – including the main graphic.

CONTACT

If you have problems, questions, ideas or suggestions, please contact one of the program contributors listed below via email.

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nuwan@iastate.edu

lgclark@iastate.edu

KNOWN BUGS

It is recommended to always click on the radio button itself to select character states instead of clicking on the text. Clicking on the text causes the program to change the first character state selection. This is an issue with the way ASP.NET Web Forms renders radio button groups and might be addressed in future ASP.NET updates. The authors will update the downloadable source code when they find a solution.

CHAPTER 6

GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE THREATENED TEMPERATE WOODY BAMBOO *KURUNA DEBILIS* (POACEAE: BAMBUSOIDEAE: ARUNDINARIEAE) FROM SRI LANKA BASED ON MICROSATELLITE ANALYSIS

A manuscript submitted to *Journal of the National Science Foundation of Sri Lanka*

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Abstract

Species of the temperate woody bamboo genus *Kuruna* Attigala, Kathriar. & L.G. Clark (Poaceae: Bambusoideae), distributed in Sri Lanka and southern India, are threatened due to deforestation and habitat fragmentation. The current study focused on the tetraploid woody bamboo *Kuruna debilis* (Thwaites) Attigala, Kathriar. & L.G. Clark, using twelve variable microsatellite loci to assess genetic diversity and population structure in six known Sri Lankan populations. Due to the rarity of the species, exhaustive sampling of accessible plants resulted in a total of only 28 individuals.

Nonetheless, allelic diversity was high at most loci and, given the limited distances separating populations (<65 km apart), they exhibited fairly high genetic differentiation (F_{ST} = 0.113) and strong isolation by distance. STRUCTURE, neighbor-joining, and Neighbor-Net analyses concur in grouping the six *K. debilis* populations into three genetic clusters consistent with the spatial proximity of populations. Due to multiple indicators of high allelic diversity, the population from the northern Horton plains (LA124) should be targeted for conservation. Moreover, the population found at Adam's peak (LA159) is also genetically important and critical to the conservation of these species due to its unique genetic diversity. As the first population genetics study of Bambusoideae in Sri Lanka, we anticipate that our results will provide a foundation for future comparative population genetics and conservation studies in the country.

Keywords: *Kuruna debilis*, microsatellites, population genetics, Sri Lanka

Introduction

Bamboos (subfamily Bambusoideae, Poaceae) are an essential component of forest and tropical high altitude grassland ecosystems worldwide (Soderstrom & Calderón, 1979; Judziewicz *et al*., 1999; Clark *et al*., 2015). In Sri Lanka, bamboos occur naturally in all three major climatic zones (wet, dry and intermediate), however, no native bamboo is found in extremely dry areas (Kariyawasam, 1998). Bamboo, in general, is an economically, culturally and ecologically important plant for Sri Lanka (De Zoysa, 1994; Gunatilleke *et al*., 1994). Most of the non-native bamboos are used in housing and constructions due to their enduring, versatile and highly renewable nature. There are no statistics on bamboo consumption, but the forestry sector master plan (FSMP) of Sri

Lanka estimated that total annual consumption was at least 80000 m^3, i.e. about 700000 culms two decades ago (FSMP, 1995). Although the native bamboos are not of economic importance, their ecological value is significant. One such example is the animal biodiversity associated with the native bamboos such as Sambar deer, many insects and fungi (Abayasinghe *et al*., 2014; *personal observations*, 28 May-04 June, 2010). Bamboo studies conducted in Sri Lanka have mainly focused on reproductive ecology (Ramanayake & Yakandawala, 1995; Ramanayake & Yakandawala, 1998; Ramanayake & Weerawardene, 2003), vegetative propagation (Ramanayake *et al*., 2001; Ramanayake *et al*., 2007) and growth and development (Rajapakse, 1992; Ramanayake *et al*., 2001).

Bamboos, with over 1500 species worldwide, are classified in three tribes, the tropical woody Bambuseae, the temperate woody Arundinarieae and the herbaceous Olyreae (Clark *et al*., 2015). A recent study revealed that all of the native Sri Lankan temperate woody bamboos along with south Indian species form a major lineage in the Arundinarieae resulting in the recognition of the genus *Kuruna* (Attigala *et al*., 2014). To date, there are seven species in this gnus including two endemic Sri Lankan species (*K. scandens* (Soderstr. & R.P. Ellis) Attigala, Kathriar. & L.G. Clark and *K. serrulata* Attigala Kathriar. & L.G. Clark), one species, *Kuruna wightiana* (Nees) Attigala, Kathriar. & L.G. Clark, endemic to south India and four species (*K. debilis*, *K. densifolia* (Munro) Attigala, Kathriar. & L.G. Clark, *K. floribunda* (Thwaites) Attigala, Kathriar. & L.G. Clark and *K. walkeriana* (Munro) Attigala, Kathriar. & L.G. Clark) occurring in both Sri Lanka and South India (Seethalaksmi & Muktesh Kumar, 1998; Muktesh Kumar, 2011; Attigala *et al.*, 2014; Attigala *et al*., in press).

The indigenous flowering plants of Sri Lanka include about 3156 species (Wijesundara *et al*., 2012). Nearly one fourth of these are endemic and concentrated in the humid southwestern quarter of the country (Gunatilleke & Gunatilleke, 1990). For many years, forests in Sri Lanka have been cleared both legally and illegally due to rapidly increasing demand for land for settlement schemes, timber production, economic and agricultural developments and weak enforcement of land use policies in Sri Lanka (Gunatilake, 1998; Government of Sri Lanka, 2000; Bandaratillake & Fernando, 2003). Several studies report that the closed-canopy forest cover has decreased from 84% in 1884 to approximately 19% in 2005 (Nanayakkara, 1996; FAO, 2005). Thus, this deforestation can negatively influence the survival of the threatened and endemic flora and fauna in Sri Lanka. Many plant molecular studies have shown that habitat fragmentation and small population size may negatively influence the genetic diversity of populations (Ellstrand & Elam, 1993; Fenster & Dudash, 1994; Fischer & Matthies, 1998; Luijten *et al*., 2000; Paschke *et al*., 2002). Low levels of genetic diversity typically limit the ability of a population to adapt to adverse environmental conditions or increased competition (Fischer *et al*., 2000; Pluess & Stocklin, 2004). Each of the *Kuruna* species found in Sri Lanka has a limited distributional range (populations <65 km apart) and therefore may be under specific threat due to this deforestation and habitat fragmentation. Of the six native *Kuruna* species, only *K. debilis* has several, spatially distinct populations in the understory of the upper cool mountain slopes of Sri Lanka's Central Province (elevation: 1500 - 2500 m). Muktesh Kumar (2011), reports that *K. debilis* has also been located recently from the Kerala part of the Western Ghats, but provides no documentation. The other five species (*K. densifolia*, *K. floribunda*, *K. scandens*, *K.*

serrulata and *K. walkeriana*) are each restricted to 1-2 populations on a few mountain summits and in open montane grassland in Sri Lanka (Figure 1). Due to their restricted distribution and habitat loss related to human activities, these five species are already at risk. Population genetics studies are essential for planning conservation strategies for these *Kuruna* species. Such studies provide conservation managers with significant information concerning elevated levels of random genetic drift and inbreeding, and reduced inter-population gene flow, and genetic estimates of these processes could potentially be used to initiate conservation planning (Ellstrand & Elam, 1993; Young *et al*., 1996).

The primary objective of this study was to assess the genetic diversity and population structure in six natural populations of the tetraploid woody bamboo *K. debilis* in Sri Lanka. The results of the current study have been presented as a poster at Botany 2014, Boise, Idaho, USA.

Methods and Materials

Population sampling

Leaf samples were collected from 28 individual plants from 6 different geographic localities, mainly from three remote montane forests and a single open montane grassland (Figure 1). *Kuruna debilis* has a pachymorph rhizome system resulting in densely packed culms that can occupy a relatively large area. Due to this clonal propagation, it is difficult in the field to differentiate individuals at a particular locality because a simple ‘‘head count’’ may not reveal the true number of genets, or genetic individuals, in a population. Therefore, samples were collected from individuals

that were readily distinguishable as a single plant and all propagating clones of an individual plant were considered as a single entity. Although the total sample of 28 individuals is relatively small, it represented all accessible plants found at the six study locations and, as shown below, was sufficient to allow for a number of statistically significant genetic results.

Marker selection

Microsatellite sequences (SSRs) are highly polymorphic and readily replicable markers consisting of short runs of tandem repeat sequence motifs evenly distributed throughout eukaryotic genomes. Based on previous temperate woody bamboo studies employing microsatellites (Kitamura *et al*., 2009; Zhan *et al*., 2009), 25 primer pairs were tested with a subset of samples to evaluate successful amplification. Of the 25 microsatellite primers 12 variable microsatellite loci were selected for the study. DNA primers used for the 12 markers are listed in Table 1. All PCR and cycle-sequencing reactions were performed in an MJ Research PTC-200 thermal cycler. PCR were performed in 25µL volumes. Amplification products were cleaned using polyethylene glycol (PEG 6000) precipitation to remove unincorporated primers and dNTPs from the PCR products. Genotyping of individual microsatellite loci were subject to standard error checking procedures, as described in DeWoody *et al*. (2006).

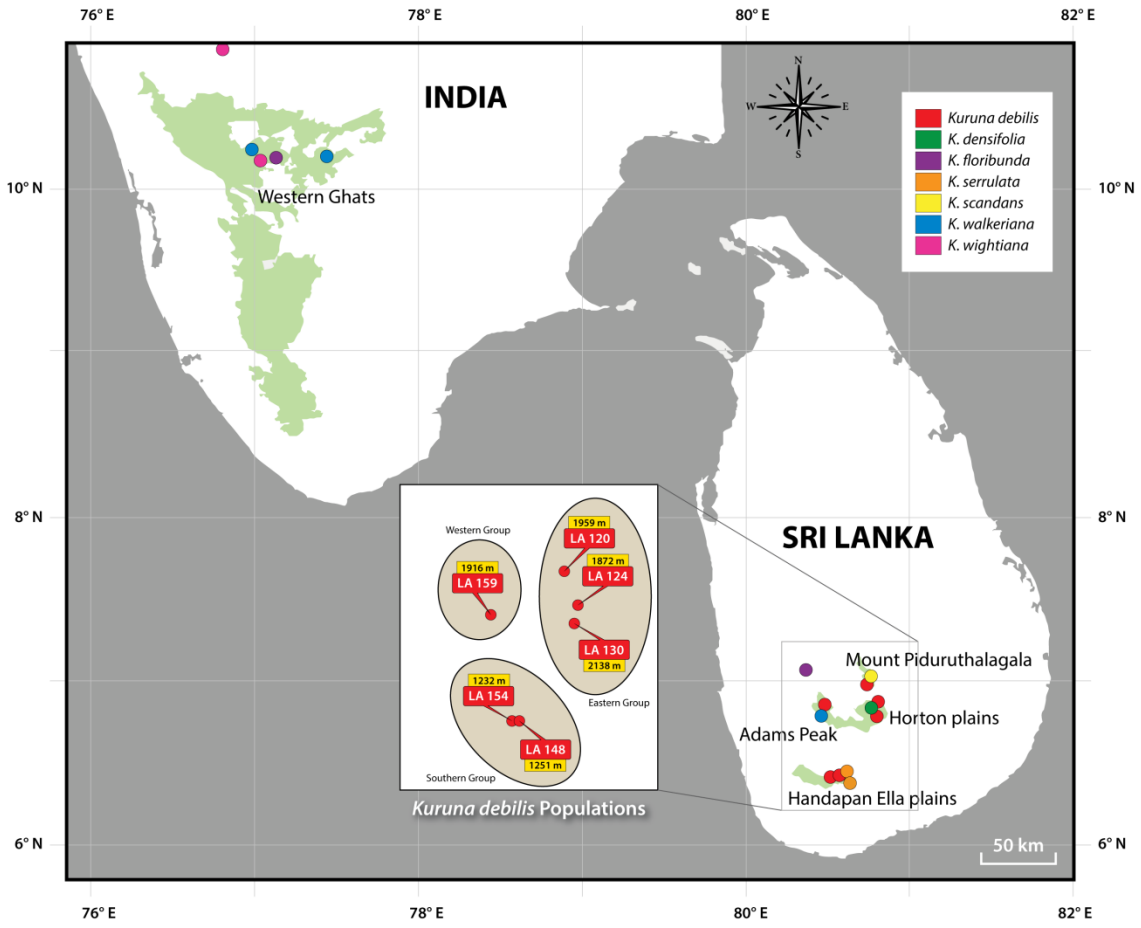

Figure 1. Distribution of Sri Lankan *Kuruna* species. Colors indicate different *Kuruna* species and the numbers in each box indicate the elevation for that population and the six *K. debilis* populations with their genetic clustering. [Map source: Wikipedia (http://en.wikipedia.org/wiki/Sri_Lanka#mediaviewer/File:Topography_Sri_Lanka.jpg)].

DNA extraction and Genotyping

Total genomic DNA extractions were performed from silica gel-dried specimens using the Iowa State University DNA Facility's Autogenprep 740 DNA extraction robot. Genotyping was performed on an ABI 3730 DNA analyzer (Perkin-Elmer, Applied Biosystems Division, Norwalk, Connecticut) by the DNA Sequencing Facility at Iowa State University. Individual tetraploid genotypes were scored from the electropherograms following the Microsatellite DNA Allele Counting-Peak Ratios (MAC-PR) method of Esselink *et al*. (2004) using GeneMapper® v4.1 (Applied Biosystems) software.

Genetic data analysis

The software SPAGeDi 1.4c (Hardy & Vekemans, 2002) was used to compute the percentage of missing genotypes and the following estimates of genetic diversity: the number of alleles per locus (N_A), the effective number of alleles (NAe) estimated following Nielsen *et al*. (2003), rarefied allelic richness (A_R) expressed as the expected number of alleles among k gene copies (12 gene copies for the current study) and the expected heterozygosity corrected for sample size (H_e). These measures and the inbreeding coefficient (F_{IS}) were estimated for each locus and each *K. debilis* population. The A_R used in the current study is corrected for variation in sample size by using the rarefaction method recommended for uneven sample sizes (Hurlbert, 1971; Petit *et al*., 1998). Further, global F-statistics (F_{IT}, F_{IS} and F_{ST}) were estimated over all loci and populations using SPAGeDi 1.4c (Hardy & Vekemans, 2002) with the significance of average values determined by permutation (10000 replicates). The observed heterozygosity (H_o) was calculated manually for each population over all loci.

Spatial genetic structure was inferred using a Bayesian clustering approach, which was implemented in STRUCTURE 2.3.4 (Pritchard *et al*., 2000). The model parameters were set to admixture with correlated allele frequencies between populations and 20 replicated runs were performed for each value of K (the number of clusters) spanning from 1 to 5. The burn-in was set to 100000 followed by 200 000 recorded Markov chain Monte Carlo steps. Each run estimated the log probability of data, L(K). Following Evanno *et al*. (2005), differences in log L(K) for successive values of K (ΔK) were used to determine the most likely number of clusters, a process implemented using the Structure Harvester (Earl & vonHoldt, 2012). The preferred value of K using this method

is the one associated with the highest value of ΔK. The K repetitions were permuted in the software CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and results were graphically represented using the software DISTRUCT 1.1 (Rosenberg, 2004).

Population differentiation was assessed by Analysis of Molecular Variance (AMOVA) using the software ARLEQUIN v3.5.1.2 (Excoffier *et al*., 2005). The K optimal genetic clusters detected with STRUCTURE determined the hierarchical levels in the AMOVA analysis and resulted in three estimates of genetic differentiation: Φ_{ST}, genetic differentiation among subpopulations relative to the total population, Φ_{SC}, genetic differentiation among subpopulations within a genetic clusters, and Φ_{CT}, genetic differentiation among genetic clusters relative to the total population. The significance of Φ-values was determined by permutation (10000 replicates). Isolation by distance (IBD) was evaluated by assessing the correlation between the log transformed genetic distance measure $F_{ST} / (1-F_{ST})$ (Rousset, 1997) and geographic distance, with significance determined by the Mantel test in the program IBDWS 3.22 (Jensen *et al*., 2005). For the IBD analyses, geographic distances were calculated manually. Significance was based on 10000 permutation replicates. For both AMOVA and IBD analyses we treated the tetraploids as diploids (Saltonstall, 2003) as the analysis programs allowed only diploid or haploid data and there was no evidence of inbreeding within populations of *K. debilis*. The diploid data matrix was generated by randomizing the genotypes within each population.

Table 1. Microsatellite markers used for PCR amplification.

Locus	Repeat Motif	Primer sequence (5'–3')	5'-Label	Allele size range (bp)	PCR parameters	Reference
Sasa500	$(AT)_n$	F: GCAGATTGCCGGTTGTTTAG R: GGAGGGCCAAGAGGTTACA	PET	297-355		
Sasa718	$(CT)_{1n}$	F: CCCTGCAACCTTCACTCCTACA R: CCCTGCAACCTTCACTCCTACA	VIC	132-144	95^0C,15 min; 35x (94 ^{0}C, 30 s,; 57 ^{0}C, 90 s, 72 ^{0}C, 60 s); 60 ^{0}C, 30 min.	Kitamura *et al*., 2009
Sasa11E	$(AC)_n$	F: $(TC)_6(AC)_5$ R: ATATTGTTTGCCTGACCTACA	FAM	268-337		
FAN11	$(AG)_n$	F: GCAATCGCGGAGTAAAGAA R: TAAGCACACAGCAGCCAGTAGG	HEX	177-187	94 °C, 3 min; 35x (94 °C, 15 s, 58 ^{0}C, 30 s, 72 °C, 45 s); 72 °C, 7 min.	
FAN20	$(AG)_n$	F: GAGGGCGAGAGGTTTGAGGAATGG R: AGGACGAACGGAGGAGGAAGCACT	HEX	137-141	94 °C, 3 min; 35x (94 °C, 15 s, 66 ^{0}C, 30 s, 72 °C, 45 s); 72 °C, 7 min.	
FAN21	$(AG)_n$	F: CGATACTACTAGCTGGGAGGAAG R: GAGGAAAGCGAACACCAGC	FAM	164-190	94 °C, 3 min; 35x (94 °C, 15 s, 62 ^{0}C, 30 s, 72 °C, 45 s); 72 °C, 7 min.	
FAN27	(AG)n	F:ACCCACAAGGGAGAGAGAG R:TCCTTCCCATTTCGGAGCC	HEX	14-152	94 °C, 3 min; 35x (94 °C, 15 s, 58 ^{0}C, 30 s, 72 °C, 45 s); 72 °C, 7 min.	Zhan *et al*., 2009
FAN26	(AC)n(AG)n	F:CGTTCCAGCGCTTCCA R:GTCCACCCACGCCTTCAC	FAM	152-168		
FAN16	$(AAG)_n$	F:CAGAGCTTCTGCCATTCCTTC R: GTTGTCCACCATCAGACGC	FAM	223-226		
FAN28	(CA)n(TG)n	F: TCCAAACCCTAATCCCCTTCAATC R: ACCCGGTCGCAACTTATCCACT	FAM	94-108	94 °C, 3 min; 35x (94 °C, 15 s, 60 ^{0}C, 30 s, 72 °C, 45 s); 72 °C, 7 min.	
FAN29	(CCG)n	F:ACGAATCCCAAGCCTCCTC R:TTGCCAACGTCTTCTGTGC	FAM	135-149		
FAN30	(TG)n	F: CTTCGCGTTTGGTTTTCTGTCCTT R: TGCGGCCAAAACTACTCCCTAATC	HEX	97-101		

A rooted majority rule consensus tree was constructed using the neighbor-joining (NJ) method with Cavalli-Sforza and Edward's chord distance (Cavalli-Sforza & Edward, 1967). The distances and the NJ tree were generated using a combination of the SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE modules in PHYLIP v. 3.695 (Felsenstein, 1989). The temperate woody bamboos: *Oldeania alpina* (K. Schum.) Stapleton and *Chimonocalamus montanus* J.R. Xue & T.P. Yi were used as outgroups to root the NJ tree. The resulting NJ tree was visualized with FigTree software v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

As a further means of visualizing the genetic clustering of sample populations, a network-building distance-based algorithm (Neighbor-Net) was performed with SplitsTree4 v. 4.13.1 (Huson and Bryant, 2006) based on the Cavalli-Sforza and Edward's chord distance obtained from GENDIST in PHYLIP v. 3.695 (Felsenstein, 1989).

Results

Allelic variation at microsatellite loci

The twelve microsatellite loci assayed were all polymorphic, and the number of alleles detected for each locus varied between 3 (FAN30) to 20 (Sasa500) leading to 94 alleles observed in total with a 2.7% missing genotypes. These selected loci on average generated 8 alleles per locus and an expected heterozygosity of 0.708. Table 2 summarizes the genetic diversity of the 12 microsatellite loci.

Genetic variation within populations

Despite the small number of plants available within populations (3-5 individuals) measures of average genetic diversity were relatively high (Table 3). For individual populations, the mean per-locus number of alleles, rarefied allelic richness, and effective number of alleles were N_A = 4.08 (range 3.33-4.75), NAe = 3.67 (2.74-4.25), and A_R = 3.75 (3.09-4.32). Populations 1 (LA120) and 6 (LA159) consistently had the lowest levels of allelic diversity, while values for populations 2 (LA124) and 5 (LA 154) were consistently the highest. Similarly, for each population, the average observed heterozygosity (H_O) and the average expected heterozygosity corrected for sample size (H_e) were 0.758 (range 0.633-0.834) and 0.708 (range 0.533-0.712) respectively. Except for the population in Adam's peak (LA159), all the other populations showed deficits in heterozygotes (average F_{IS} = 0.170; range 0.175-0.012). However, the LA159 population showed an excess of heterozygotes [F_{IS} = -0.024 (Table 3)].

Table 2: Locus-level and average measures of genetic diversity for twelve microsatellite loci genotyped across six *K. debilis* populations (N_A: number of alleles with non-zero frequency, NAe: effective no of alleles, H_e: expected heterozygosity corrected for sample size, H_O: observed heterozygosity).

Locus	Sample size	% of missing genotypes	N_A	NAe	H_e	H_O
Sasa500	28	0	20	7.71	0.871	0.678
Sasa718	28	0	7	5.40	0.815	0.900
Sasa11E	28	0	4	3.15	0.683	0.967
FAN11	28	3.6	6	4.95	0.798	0.845
FAN16	28	7.1	2	1.88	0.468	0.567
FAN20	28	0	7	3.33	0.700	0.678
FAN21	28	0	10	5.99	0.833	1.000
FAN26	28	0	9	4.11	0.757	0.870
FAN27	28	21.4	7	3.49	0.714	0.767
FAN28	28	0	7	4.60	0.783	1.000
FAN29	28	0	12	4.86	0.794	0.588
FAN30	28	0	3	1.40	0.284	0.245
Average over all loci	28	2.7	7.83	4.24	0.708	0.758

Table 3. Measures of genetic diversity for each the 6 populations of *K. debilis* averaged across 12 microsatellite loci. (N_A: number of alleles with non-zero frequency, A_R: rarefied allelic richness, NAe: effective no of alleles, H_e: expected heterozygosity corrected for sample size, H_O: observed heterozygosity, F_{IS}: inbreeding coefficient).

Population	Locality	Sample size	% of missing genotypes	N_A	NAe	A_R	H_e	H_O	F_{IS}
Population 1 (LA120)	Piduruthalagala mountain	5	1.7	3.33	2.74	3.09	0.533	0.633	0.175
Population 2 (LA124)	Horton Plains (north)	5	6.7	4.75	4.25	4.32	0.695	0.767	0.152
Population 3 (LA130)	Horton Plains (south)	5	1.7	4.50	3.81	3.93	0.632	0.750	0.042
Population 4 (LA148)	Handapanella mountain	3	0	3.83	4.01	3.83	0.712	0.834	0.119
Population 5 (LA154)	Handapanella mountain	5	1.7	4.67	4.25	4.17	0.665	0.800	0.012
Population 6 (LA159)	Adam's peak	5	3.3	3.42	2.94	3.18	0.570	0.767	-0.024
Multilocus Average: all populations combined		28	2.7	7.83	4.24	4.58	0.708	0.758	0.170

Population genetic structure

The proportion of the observed genetic variation between clusters ranged from F_{ST} = -0.053 for locus FAN27 to 0.394 for locus FAN30 with an average value 0.113 that was significantly great than zero ($P < 0.001$; Table 4). Individual locus estimates of the inbreeding coefficient (F_{IS}) ranged from -0.055 (FAN26) to 0.414 (FAN29) with a mean of 0.078 ($P < 0.001$; Table 4).

STRUCTURE analyses using the Evanno method in Structure Harvester grouped the 6 populations into K = 3 clusters (Figures 2, 3A). The estimated population structure inferred for K = 2-5 is shown in Figure 3A. The three clusters correspond well to the geographic distribution of the populations (Figure 1), with Populations 1, 2 and 3 (LA120, LA124 and LA130 forming an "Eastern" cluster, populations 4 and 5 (LA148 and LA154) a "Southern" cluster, and population 6 a "Western" cluster (Figure 3A). The

populations in the Eastern cluster were sampled from Piduruthalagala mountain (LA120) and the Horton plains (LA124 and LA130). These two localities are in relatively close proximity and are separated by ca. 15 km. The two populations in the Southern cluster are from Mount Handapanella.

Table 4. Individual locus and average F-statistic measures estimated across six populations of *K. debilis*. (F_{IT}: fixation index as the global population; F_{IS}: inbreeding coefficient in relation to subpopulations; F_{ST}: inbreeding due to differentiation of subpopulations in the total population). Significance of average F-statistic estimates: *, **, and *** denote p-values less than 0.05, 0.01, and 0.001, respectively.

Locus	F_{IT}	F_{IS}	F_{ST}
Sasa500	0.417***	0.251***	0.221***
Sasa718	-0.012	-0.099**	0.079**
Sasa11E	-0.039	-0.103	0.058*
FAN11	0.148***	-0.006	0.153***
FAN16	0.250**	0.163	0.103
FAN20	0.377***	0.312***	0.095*
FAN21	-0.009	-0.112**	0.092***
FAN26	0.082	-0.055	0.130***
FAN27	0.095	0.140*	-0.053
FAN28	0.043	-0.013	0.055*
FAN29	0.508***	0.414***	0.161
FAN30	0.531***	0.225**	0.394***
Average	**0.183*** **	**0.078*** **	**0.113*** **

Genetic structure inferred from AMOVA and genetic distances

Partitioning of genetic variability by AMOVA revealed that 8.35% and 7.52% of the total genetic variation was distributed among the K = 3 clusters recognized by STRUCTURE analysis, and among populations within these clusters, respectively. Both of these values were significantly greater than zero ($P < 0.001$; Table 5). The remaining ca. 84% of the variation was distributed among individuals within populations.

Table 5. AMOVA analysis of genetic differentiation among the K = 3 clusters recognized by STRUCTURE analysis and among populations within these clusters. Significance of estimated phi-statistics (see text): *, and *** denote p-values less than 0.05 and 0.001, respectively.

Source of variation	df	SSD	Variance components	% of variance	Φ_{ST}	Φ_{SC}	Φ_{CT}
Among clusters	2	38.159	0.314	8.35			
Among populations within clusters	3	25.069	0.283	7.52			
Within populations	106	335.817	3.168	84.13			
Total	111	399.045	3.765		**0.159***	**0.082*****	**0.084*****

The Mantel test of the correlation between Rousset's genetic distance and geographic distance indicated strong and highly significant isolation by distance among the six *K. debilis* populations ($r^2 = 0.4016$; $P = 0.0024$) (Figure 3D).

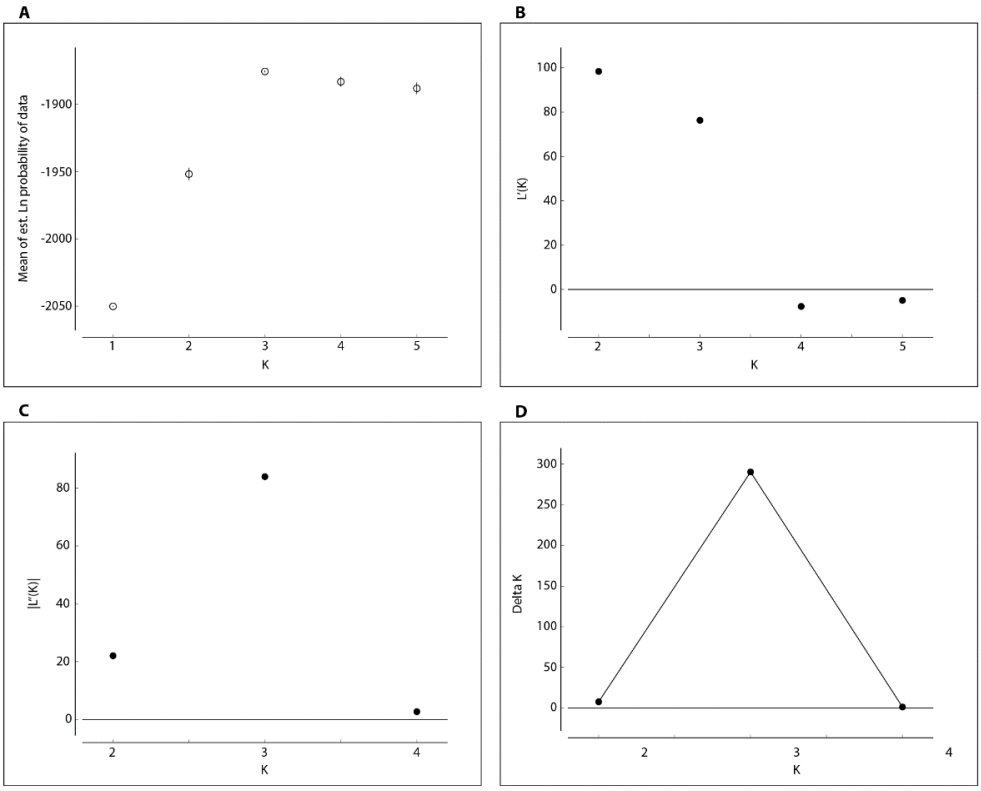

Figure 2. Structure Harvester results of STRUCTURE analyses for K=2–5 putative genetic clusters of *K. debilis* individuals. A: Mean L(K) (± SD) over 20 runs for each K value. B: Rate of change of the likelihood distribution (mean ± SD) calculated as L′(K) = L(K) – L(K– 1). C: Absolute values of the second order rate of change of the likelihood distribution (mean ± SD) calculated according to the formula: |L″(K)| = |L′(K + 1) –L′(K)|. D: ΔK calculated as ΔK = mean |L″(K)|/sd[L(K)]. The modal value of this distribution is the most probable number of clusters or the uppermost level of structure, here three clusters.

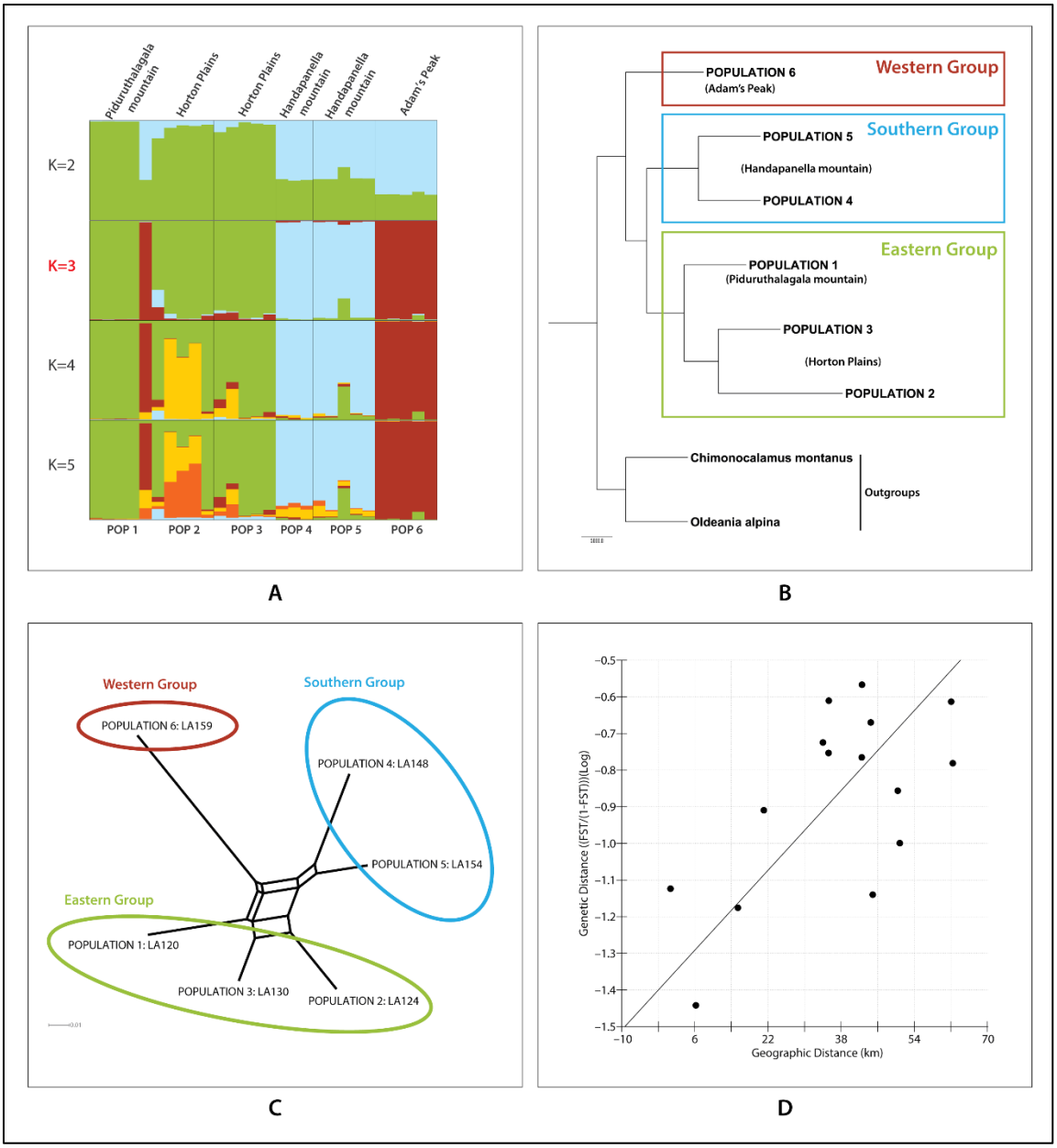

Figure 3. Analyses of genetic structure among the 6 K. debilis populations. A: Bayesian clustering using STUCTURE for K= 2-5 putative genetic clusters. Each individual is represented by a vertical column and the populations are separated by a vertical black line. Different colors in the same column for each individual indicate the percentage of estimated membership in a cluster. B: Rooted Neighbour-joining (NJ) tree based on Cavalli–Sforza and Edwards' chord distance. C: Neighbor-Net network showing genetic relatedness among the study populations based on Cavalli-Sforza and Edward's chord distance. D: Isolation by distance plot of Rousset's genetic differentiation on geographical distance (km).

Genetic relationships within the geographic groups

The rooted NJ tree for the six *K. debilis* populations (Figure 3B) resulted in three clades corresponding to the three genetic clusters indicated by the STRUCTURE analysis (Figure 3A). The Neighbor-Net network derived from the SplitsTree analysis also revealed the same three population genetic clusters (Figure 3C).

Discussion

Locus and population level genetic diversity

Of the six *K. debilis* populations sampled for this study, population 1 (LA120), which was collected from mount Piduruthalagala, was unusual in displaying lower allelic and genetic diversity compared to the other five populations (Table 3). The lower diversity for this population is not due to a smaller sample size or more missing loci, as these were similar to the other populations examined. The 2.7% of missing genotypes is due to failure to amplify certain loci.

Of the various dimensions of genetic diversity, allelic richness is often considered to be of key relevance in conservation programs (Petit *et al*., 1998; Simianer, 2005; Foulley & Ollivier, 2006). Allelic diversity is particularly sensitive to bottlenecks in population size and genetic drift, and may be an important indicator of a population's adaptive potential, as the limit of selection response is mainly determined by the initial number of alleles regardless of the allelic frequencies (Hill & Rasbash, 1986). Based on these expectations, Petit *et al*. (1998) regarded allelic richness as the most informative measure of genetic variation for identifying populations for conservation. From this perspective, population 1 may be of less value for conservation than populations 2–6.

Vekemans and Hardy (2004) surveyed fine-scale spatial genetic structure analyses in plant populations based on both allozymes and microsatellites and showed that, on average, outcrossing species have an F_{IS} value of 0.014. The global F_{IS} value of 0.078 (Table 4) we obtained for *K. debilis* is significantly low and indicates that these populations are primarily outcrossing.

Genetic structure of *K. debilis* populations

Genetic differentiation among angiosperm populations arises due to a variety of factors. Factors associated with relatively low values of genetic differentiation include woody habit, outcrossing breeding systems and wind dispersal (Hamrick *et al*., 1992), all of which are characteristic of *K. debilis*. Based on Wright's (1978) qualitative guidelines for the interpretation of F_{ST}, our global value for *K. debilis* (F_{ST} = 0.113) is within the suggested range for moderate genetic differentiation (range: 0.05-0.15). That said, it is rather high given the relatively close spatial proximity of the study populations.

This study showed that *K. debilis* populations cluster into three genetically distinct sub-groups in the central mountains of Sri Lanka: one specific to the east (populations LA120, LA124 and LA130), one specific to the south (populations LA148 and LA154) and a single unique population to the west (LA159). These groupings were evident from all three of the clustering analyses used (STRUCTURE, neighbor joining, and Neighbor-Net). The observation that the number of genetic clusters is lower than the number of populations is indicative of partial barriers to gene flow, which may be either historical or ongoing (Vergara *et al*., 2014). Also consistent with this observation, the Mantel test shows that geographic distance explains 40% of the genetic distance between population pairs. This positive association between geographic distance and genetic

distance suggests that migration rates between these *K. debilis* populations decrease with increasing distance as expected under an isolation by distance (IBD) model of gene flow (Wright, 1943).

Conservation implications

This analysis is the first study investigating the genetic diversity and structure of *K. debilis* from Sri Lanka, with samples from almost all known populations evaluated using microsatellite markers. Although several studies have previously quantified population level genetic variation in bamboos, none of them have related this variation to the conservation of bamboo populations or species (Nayak *et al*., 2003; Ramanayake *et al*., 2007; Lalhruaitluanga & Prasad, 2009; Mukherjee *et al*., 2010; Triplett *et al*., 2010). Populations 2–5 all showed generally high levels of genetic diversity (Table 4). Population 2 (LA124), from the northern part of the Horton plains, showed the highest levels of N_A, NAe, A_R and is a particularly good candidate for conservation. The single population from Adam's peak (population 6: LA159) formed its own genetically distinct cluster is also a potential candidate for conservation due to its unique genetic diversity. As the six populations of *K. debilis* formed three genetically identifiable clusters, we recommend that populations separated by more than ca. 35 km (as is the case for these clusters) should be treated as distinct units for management and conservation, while those within ca. 15 km should be managed jointly. These recommendations serve as an initial step towards identifying management units for threatened bamboos in Sri Lanka. However, since our samples sizes are relatively lower one must use these results cautiously.

Despite the small sample sizes, levels of genetic variation were found to be relatively high within individual populations. Further, tests of genetic differentiation among populations were found to be highly significant. Consequently, this study provides important insight into the genetic diversity and connectivity of *K. debilis* and is a significant initial step towards the conservation management of this threatened temperate woody bamboo species. For these rare and threatened species with limited distribution areas, a single stochastic event, such as a serious insect attack or pathogen infection, could cause catastrophic reductions in population size and genetic diversity, and even extinction (Ma *et al*., 2013). Taking proper measures to protect their current populations is required.

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CHAPTER 7

GENERAL CONCLUSIONS

The research presented in this dissertation focuses on the evolutionary history of the temperate woody bamboos (Poaceae: Bambusoideae: Arundinarieae) with an emphasis on the Sri Lankan temperate woody bamboos. In addition, this study focuses on the morphology of this group and the population diversity of one of the Sri Lankan temperate Arundinarieae species. The subfamily Bambusoideae (Poaceae) includes mainly forest grasses that comprise 119 genera and approximately 1,480 species (Bamboo Phylogeny Group [BPG] 2012; Clark et al. 2015) classified into two tribes of woody bamboos (the tropical Bambuseae and the temperate Arundinarieae) and one tribe of herbaceous bamboos (the Olyreae). The temperate woody bamboos (Arundinarieae) are a diverse clade of 31 genera and ca. 546 species distributed primarily in forests of the northern temperate zone, but are also found at high elevations in tropical regions (Asia, high elevation habitats of Africa, Madagascar, India and Sri Lanka) (Clark et al. 2015). Even though Arundinarieae has a difficult taxonomy and morphological synapomorphies have yet to be identified for the tribe, many molecular studies strongly support the monophyly of this tribe (BPG 2012; Kelchner et al. 2013; Attigala et al. 2014). Thus my dissertation work incorporates both conventional taxonomic and modern molecular techniques to understand the relationships among the clades within Arundinarieae. The major findings of this work include:

I. A new temperate woody bamboo genus, *Kuruna*, from Sri Lanka is recognized based on chloroplast sequence data from five markers (coding:

ndhF 3' end; non-coding: *rps16-trnQ*, *trnC-rpoB*, *trnD-trnT*, *trnT-trnL*). This genus represents the twelfth major lineage of Arundinarieae and is characterized by pachymorph culm bases (= pachymorph rhizomes) with short necks, unicaespitose clumps, culm leaf girdles ca. 1 mm wide, usually abaxially hispid culm leaves with non-irritating hairs, persistent foliage leaf sheaths, complete branch sheathing and acute to biapiculate palea apices. Analyses of the combined dataset strongly support the monophyly of this new lineage.

II. A complete taxonomic treatment is prepared for *Kuruna*. This genus is distinguished by a combination of vegetative and reproductive characters including features of rhizomes, branching and synflorescences (see I.). Seven species, distributed in Sri Lanka and southern India, are included in this genus: *Kuruna debilis*, *K. densifolia*, *K. floribunda*, *K. scandens*, *K. walkeriana*, *K. wightiana* (transferred to *Kuruna*) and the newly described *K. serrulata*. This revision includes an updated description of the genus, detailed descriptions and a preliminary assessment of conservation status for all seven species, line illustrations for all species, and a morphological key for their identification.

III. Phylogenetic relationships among the twelve major lineages of Arundinarieae are explored based on sequences of complete plastomes and three low copy nuclear genes. Most of the previously recognized major clades are supported by both data sets, though there are some conflicting phylogenetic signals. Plastome data revealed all twelve major clades within Arundinarieae. The

low-copy nuclear gene phylogeny resolved most of the previously recognized major lineages of Arundinarieae such as clades I, II, III, VI, VII and XII except for clades IV and V. Further, low-copy nuclear gene phylogeny revealed Clade XII as the early diverging lineage of Arundinarieae and a sister relationship between the clades IV and V. In addition, Analyses of morphological character evolution of rhizomes and reproductive structures revealed that, pachymorph rhizomes could be the ancestral state while the leptomorph rhizomes probably evolved multiple times in Arundinarieae and also in Bambusoideae. Further, the pseudospikelets evolved independently multiple times during the evolution of Arundinarieae.

IV. A web-based multi-access interactive identification tool is developed; it enables users with plant material from an unknown species of *Kuruna* to visually inspect characteristics of the bamboo and identify it as one of seven species in the genus. The images used in the interactive key ensure that the characters and unique character states are easy to understand. Furthermore, this application can easily be modified to develop interactive keys for other plant groups, especially bamboos, as the example database design already includes bamboo characters and character states.

V. Genetic diversity and population structure in six natural populations of *Kuruna debilis* are assessed based on twelve variable microsatellite loci. A moderate level of genetic differentiation among populations is revealed and showed evidence of isolation by distance. Population structure analyses

grouped the six *K. debilis* populations into three genetic clusters suggesting that there is gene flow between populations within each genetic cluster.

These findings represent the most recent understanding of the temperate woody bamboos and serve as the best understood phylogenetic framework for further studies of, for example, the role of hybridization and reticulate evolution or large-scale biogeography and morphological character evolution in the tribe. The population genetic analysis of *K. debilis* is the first population genetics study of Bambusoideae in Sri Lanka, and these results will provide a foundation for future comparative population genetics and conservation studies in the country.

Future Directions

Though the plastid topology that we obtained was not completely resolved, it can still serve as a foundation for testing biological or biogeographic hypotheses. The incorporation of more polymorphic markers into molecular analyses is necessary to obtain better resolution in order to critically examine divergence times, biogeography and morphological evolution within temperate woody bamboos. Very little is known about the biogeography or vicariance vs. dispersal events and mechanisms that produced the current distribution patterns of bamboos. Further, rigorous study of the south Indian *Kuruna* species, especially inclusion of these species and more African alpine species in a molecular study is critical to better understand the biogeography of Arundinarieae. The current plastome phylogeny could be used as the basis for molecular dating analyses and ancestral character state reconstruction which will provide insights about biogeography and morphological character evolution in Arundinarieae.

However, due to some of the inconsistent and complex results obtained from the low-copy nuclear data, we suggest that further studies are needed to understand especially how hybridization events impacted the evolution of the entire Arundinarieae tribe. In addition, future studies of morphological evolution are needed but these will require better sampling within *Phyllostachys* clade.

The population genetics results of the current study are an important initial step to understand the genetic diversity of these threatened temperate woody bamboo species in Sri Lanka. This study could serve as the basis for future studies leading to the implementation of conservation strategies for this important species. Further, ecological niche modeling and Geographic Information System (GIS) approaches could be applied to evaluate the conservation-related problems of Sri Lankan *Kuruna* species.

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